BONE HEALTH IN ELITE BALLET DANCERS: A MULTIDISCIPLINARY APPROACH

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Signature.....

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FOUNDING SOURCES

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ABSTRACT

Background It has been reported that dancers are at greater risk of developing low bone mineral density (BMD) compared to general population; however, some published studies also highlight the positive effects of dance training on bone metabolism. Given the existing controversy, the aim of the current work was a) to investigate bone health status of professional ballet dancers and vocational dance students, and b) to investigate associated factors and mechanisms involved in dancers' bone health. *Design* Cross-sectional, longitudinal analysis (2-yrs follow-up) and genetic association studies were conducted on a population which consisted of professional ballet dancers, vocational dance students and controls. *Methods* The total of 58 professional ballet dancers (66 sex- aged-matched controls), and 152 vocational dance students (96 aged- and sex-matched controls) were screened for BMD status at impact [femoral neck (FN); lumbar spine (LS)] and non-impact sites (forearm). Tanner staging, age at menarche and menstrual status were assessed via questionnaires. Bone mass, nutrition, peak height velocity estimation, energy availability, insulin-like growth factor I (IGF-1), oestrogens, growth hormone, and sclerostin serum concentrations were longitudinally measured in a sub-sample of 101 vocational dance students and age- and sex-matched controls. Association between polymorphisms of the Wnt/β-catenin and ER signalling pathways with low BMD were further investigated. *Results* Female vocational dance students were more likely to display low BMD at the forearm and LS than controls (OR= 0.1; $p<0.05$ and OR=0.2; $p<0.05$, respectively); the prevalence of low BMD at the forearm was significantly higher in female professional ballet dancers than controls (37.5% vs. 17.4%, $p<0.001$). During the follow-up, both female and male vocational dancers revealed significantly lower BMD at impact and non-impact sites ($p<0.001$) compared to controls. Serum IGF-1 concentrations were significantly increased in vocational dancers compared to controls at 2yrs follow-up ($p<0.05$), as well as serum sclerostin ($p<0.05$). Genetic variants at the Wnt/β-catenin and ER signalling pathways were identified as risk factors for low BMD at both impact and non-impact sites. *Conclusion* Professional dancers and vocational dance students have lower bone health compared to controls. Genetic mechanisms seem to be determinant. It is recommend that dancers performing at elite level should be referred for bone densitometry.

KEYWORDS osteoporosis; BMD; dancers; genetics; sports medicine

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LIST OF ABBREVIATIONS

BMD	Bone mineral density
GH	Growth hormone
IGF-1	Insulin – like growth factor 1
HHG	Hypothalamic – hypophyseal – gonadal
RED-S	Relative energy deficiency in sport
BMC	Bone mineral content
SNP	Single-nucleotide polymorphism
ACSM	American College of Sports Medicine
IADMS	International Association of Dance Medicine and Science
JDMS	Journal of Dance Medicine and Science
MPPA	Medical problems of Reforming Artists
RCT	Randomised controlled trials
GRADE	Grading of Recommendations, Assessment, Development and Evaluation
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
DXA	Dual-energy x-ray absorptiometry
LS	Lumbar spine
WHO	World Health Organization
RMR	Resting metabolic rate
ISCD	International Society of Clinical Densitometry
FN	Femoral neck
FM	Fat mass
LM	Lean mass
BPAQ	Bone-specific physical activity questionnaire
BMAD	Bone mineral apparent density
PHV	Peak height velocity
PTH	Parathyroid hormone

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To my Mum and Dad, I dedicate this Thesis.

RESPONSIBILITIES

- Conduct and submit for publication a systematic review;
- Study design and methodology of the original studies included in the Thesis;
- Obtain ethical approval in Portugal and UK;
- Recruit participants in Portugal and UK;
- All arrangements in Portugal and UK for data collection;
- Collect data (in all domains) in Portugal and UK;
- Analyse all the data collected (in all domains), under the supervision of my supervisors and Professor Alan Nevill (for statistical analysis);
- Data interpretation;
- Write the original studies and submit them for publication in peer-reviewed journals;
- Prepare oral presentation of the results from the original studies to be presented in scientific congresses.

SKILLS ACQUIRED AS A RESULT OF THIS PHD

- Knowledge on how to conduct systematic reviews;
- Knowledge on how to delineate research studies, how to analyse data and how to interpret scientific research results;
- Knowledge on bone physiology, bone biology and molecular genetics;
- Acquisition of genomic analysis techniques;
- Acquisition of skills in laboratory experimentation required for scientific research in biomedicine;
- Acquisition of radioimmunoassay techniques;
- Knowledge on recruiting human participants for scientific research studies;
- Writing skills;
- Oral presentation skills;
- Experience on conducting scientific research studies in different European countries (dealing with different ethics committee, different institutional processes, different participants' recruitment processes, countries' law and facilities).
- Developed abilities to scientifically communicate in two different languages (Portuguese and English).

LIST OF PUBLICATIONS

1. Articles in Academic (peer-review) Journals

Amorim T, Durães C, Metsios GS, Wyon M, Nevill A, Flouris A, Maia J, Machado JC, Gomes NT, Marques F, Adubeiro N, Nogueira NL, Koutedakis Y. Genetic variation in Wnt/β – catenin and ER signalling pathways in professional ballet dancers and vocational dance students: associations with low bone mineral density. *Submitted to Journal of Clinical Endocrinology and Metabolism*.

Amorim T, Metsios GS, Wyon M, Nevill A, Flouris A, Maia J, Machado JC, Gomes NT, Marques F, Adubeiro N, Nogueira NL, Matthews K, Koutedakis Y. Associations between nutrition, energy expenditure, and energy availability with bone mass acquisition in female and male vocational dance students: a mixed-longitudinal study. *Submitted to Bone.*

Amorim T, Metsios GS, Flouris A, Maia J, Gomes TN, Wyon M, Franklim M Machado J, Adubeiro N, Nogueira L, Koutedakis Y. Associations between oestrogens, growth hormone, and insulin-like growth factor I with bone mass acquisition in adolescents involved in rigorous training regimens: a mixed-longitudinal study. *Submitted to Journal of Bone and Mineral Research.*

Amorim T, Koutedakis Y, Metsios GS, Wyon M, Nevill A, Flouris A, Maia J, Machado JC, Marques F, Adubeiro N, Nogueira NL, Matthews K, Dimitriou L. Prevalence of low bone mineral density in vocational and professional ballet dancers. Osteoporosis International. 2017;28(10):2903-12.

Amorim T, Metsios GS, Wyon M, Nevill A, Flouris A, Maia J, Teixeira E, Machado JC, Marques F, Koutedakis Y. Bone mass of female dance students prior to professional dance training: a cross-sectional study. Plos One. 2017;12(7):1-11.

Amorim T, Wyon M, Maia J, Machado JC, Franklim M, Metsios GS, Flouris AD, Koutedakis Y. Prevalence of Low Bone Mineral Density in Female Dancers. Sports Medicine. 2015;45(2):257-68.

2. Abstracts in conferences

Amorim T, Maia J, Metsios GS, Flouris A, Wyon M, Machado J, Franklim M, Adubeiro N, Nogueira L, Koutedakis Y. "Bone mineral accrual during puberty and its association with serum levels of growth hormone and insulin-growth factor I in vocational ballet dancers". The American Society for Bone and Mineral Research Annual Meeting, 15-19/10/2016, Atlanta, USA, *J Bone Miner Res 31(Suppl 1).*

Amorim T, Durães C, Maia J, Machado JC, Nogueira L, Adubeiro N, Flouris AD, Metsios GS, Marques F, Wyon M, Koutedakis Y. "Genetic variants at the Wnt/β-catenin and oestrogen receptor signalling pathways are associated with low bone mineral density in dancers". European Calcified Tissue Society Conference ECTS 2016, Rome, Italy. *Bone Abstracts Vol **5**, P238 doi:10.1530/boneabs.5.P238, 2016*

Amorim T, Humber LH, Fonolla F R, Flouris D A, Metsios D G, Maia J, Wyon M, Koutedakis Y. "Cortical and trabecular bone analysis of professional dancers using 3D-DXA: a case control study." *Osteoporosis Int, 27(1):S1, doi 10.1007/s00198-016-3530-x, 2016.*

Amorim T, Dimitriou L, Metsios G, Wyon M, Alan N, Flouris A, Maia J, Machado J, Franklim M, Adubeiro N, Nogueira L, Matthews K, Koutedakis Y. "Prevalence of low bone mineral density, osteopenia, osteoporosis and its associated risks factors in female ballet dancers". The American Society for Bone and Mineral Research Annual Meeting, 9-12/10/2015, Seattle, USA, *J Bone Miner Res (*S187), 2015.

Amorim T, Wyon M, Metsios G, Maia J, Machado J, Franklim M, Flouris A, Koutedakis Y. "Bone mineral density and nutrition intake in dance students: an international comparison between two vocational dance schools in the UK and Portugal." The 25th *Int. Assoc. of Dance Medicine & Science* Annual Conference, 9-11/10/2015, Pittsburgh, Pennsylvania, USA, Book of Abstracts, p. 40, 2015.

Amorim T, Wyon M, Metsios G, Maia J, Machado J, Franklim M, Flouris A, Koutedakis Y. "Are female dancers at risk of developing low bone mineral density? A systematic review". The 25th *Int. Assoc. of Dance Medicine & Science* Annual Conference, 9-11/10/2015, Pittsburgh, Pennsylvania, USA, Book of Abstracts, p141, 2015.

Amorim T, Wyon M, Maia J, Machado J, Franklim M, Koutedakis Y. Bone health and vocational dance training. The 24th *International Association of Dance Medicine and Science* Annual Meeting, Basel, Switzerland, Book of Abstracts, p 34, 2014.

Amorim T, Maia J, Machado J, Franklim M, Wyon M, Koutedakis Y. Bone mineral density in active and former elite ballet dancers. The 19th *European College of Sports Sciences* Annual Conference, Amsterdam, Netherlands, Book of Abstracts, p465, 2014.

CHAPTER 1: INTRODUCTION

Skeletal health is essential for overall quality of life [1]. Bone mass increases throughout childhood, with particular acceleration during puberty [2]. This accrual is continuous, until a peak is reached during the third decade of life, after which the skeleton begins to lose its capacity to form new bone and will become increasingly fragile [3]. This tendency suggests that a higher peak bone mass will provide a better bone stock in adulthood, which is important to prevent diseases as osteoporosis [3]. Indeed, the most common skeletal disorder is osteoporosis, characterised by low bone mineral density (BMD), which leads to decreased bone strength and increased risk of fracture [4]. The diagnosis of this clinical condition is based on the World Health Organization (WHO) criteria, whereas osteoporosis is considered when bone mineral density (BMD) values lies 2.5 standard deviations (SD) or more below the average value, and osteopenia when BMD values lies between 1.0 and 2.5 SD [4]. It is estimated that 1 in 3 women and 1 in 5 men will experience an osteoporotic fracture during their lifetime [5]. A rise on the prevalence of this disease is also expected; by 2020 the prevalence of low BMD (i.e. osteopenia, which often leads to osteoporosis) is estimated to be over 47 million in the US [6]. In accordance, by 2025 and 2050 the estimated costs of fracture healing are projected to be $25.3 billion in the US [7] and £51 billion in Europe [5], respectively.

To decrease the current burden of osteoporosis it is crucial to identify and delineate preventative measures in high-risk communities. Although low BMD and osteoporosis have been traditionally associated with elderly and postmenopausal women [5, 8], some other populations, such as elite athletes, may also be at higher risk to develop these clinical conditions [9, 10]. Indeed, whereas the effects of exercise on bone metabolism are well established [1,8–10], the effects of elite sports training on bone health are still controversial due to the specificity of the activity [11,12]. For instance, professional ballet dancers and vocational dance students are involved in daily classes of several hours of weight-bearing activity [13,14]. The high levels of muscular strength required for technical performance and the weight-bearing activity associated with jumping may stimulate bone-forming cells at impact sites (femoral neck and lumbar spine) [13,15,16]. Consequently, when comparing elite dancers with non-exercising controls, it would be expected to find significantly higher BMD values at impact sites and similar BMD values at non-impact sites (forearm). However, elite dancing is also an aesthetic activity where body size is essential for performance [13]. In accordance,

literature on the topic suggests that, to correspond with the aesthetic and artistic demands, elite dancers may restrict their diet (decreasing energy and/or nutrient intake), which may lead to low body weight, menstrual disturbances, and/or negative energy balance [17–19]. It is assumed that these conditions negatively affect the growth hormone (GH)– insulin-like growth factor-I (IGF-I) axis and hypothalamic– hypophyseal–gonadal (HHG) axis [11,20–23], impairing further bone mineralization. The inter-relationship of the aforementioned factors is known as the 'female athlete triad', or 'RED-S' (relative energy deficiency in sport) [11,24]. Indeed, several studies show that female professional ballet dancers and vocational dance students are at risk for osteoporosis and have lower BMD values than non-exercising controls due to the aforementioned factors [18,19,25]. However, controversially, other studies in professional ballet dancers and vocational dance students show that BMD was not associated with body weight, menstrual disturbance nor energy balance [26,27]. Furthermore, contrary to all aforementioned findings, it has been suggested that the mechanical impact from dancing provides protection against the osteoporosis risk factors that dancers are exposed (i.e. low body weight, menstrual disturbances and low energy availability), resulting in higher BMD values than non-exercising controls [16,28,29]. Therefore, taken together, it seems that the question of whether ballet dancers are at risk of developing low BMD is unanswered. Furthermore, it also seems that the factors associated with low BMD in elite dancing are not well established; and even less is known in relation to male professional ballet dancers and male vocational dance students. Actually, current research on the topic has been focusing in female participants and on their nutritional status, menstrual, and body weight as the main causes for low BMD phenotypes. However, it should be noted that the aetiology of low BMD and osteoporosis is multifactorial [30,31]. Factors such as genetics and hormonal profiles should also be considered in studying dancers' bone health, given their well-known association with low bone mass phenotypes [30,31]; to our knowledge, these factors have not been considered previously in this population. Thus, to understand dancers´ bone health, a multidisciplinary approach is required. Only by assessing cross-sectionally and longitudinally the same group of dancers in several domains as genetics, endocrinology, anthropometry, nutrition and energy availability is possible to offer new insights on dancers' bone health and its associated factors.

It was first studied the state-of-the-art on dancers' bone health. Afterwards, it was delineated several original studies aiming at addressing some gaps and limitations on current available literature to provide new evidence on dancer's bone health. In

accordance, this PhD thesis includes: a) a cross-sectional study to assess bone mass parameters in female dance students selected for professional dance training (first year vocational dance students, before receiving dance training stimuli); b) an epidemiological study with female and male professional ballet dancers and vocational dance students to assess the prevalence of low BMD, and to investigate the association between low BMD with body mass, fat mass, lean mass, menarche and maturation; c) a longitudinal study aiming at modelling bone mineral content (BMC) and BMD accruals in female vocational dance students throughout growth, and to determine whether circulating levels of oestrogens, GH and IGF-1 are significant predictors of BMC and BMD changes throughout growth; d) a longitudinal study in vocational female and male vocational dancers to determine whether nutrition and energy availability are significant predictors of BMC and BMD changes throughout growth; and e) a genetic association study aiming at assessing if single-nucleotide polymorphisms (SNPs) in the Wnt/β-catenin and ER signalling pathways are associated with low BMD in female and male professional ballet dancers and vocational dance students.

It is expected that the multidisciplinary approach of this PhD will contribute to a wider understanding of the putative causes involved in dancers' bone mass acquisition, thus further contributing to dancers' bone health improvements. Finally, it is also expected that this thesis will provide the first insights on the topic in relation to male dancers.

CHAPTER 2: REVIEW OF THE LITERATURE
(A systematic review on the prevalence of low BMD in female dancers)

Parts of this chapter have been published in a peer-review journal entitled “Prevalence of Low Bone Mineral Density in Female Dancers”; Sports Medicine, 2015;45(2):257-68. The author of this Thesis appears as the leading author.

The purpose of this systematic review was to investigate and examine the information available in relation to prevalence and incidence of low BMD in female dancers.

INTRODUCTION

Bone mineral density (BMD) is commonly used to assess bone health, including the diagnosis of osteoporosis and prediction of bone fracture risk [32,33]. It is believed that the aetiology of a low BMD is both genetic and environmental [34], with the former to explain up to 80% of the variance, whereas the remaining 20% is modulated by environmental factors such as diet and physical activity [35]. However, only environmental factors can be possibly modified by appropriate interventions with the aim to stimulate bone mass gains [10]. Indeed, it has been found that participation in various physical activities is associated with positive effects on bone mineral accrual [9,10,36]. Weight-bearing activities seem to be the most effective for bone mass increases [37,38], which, nevertheless, seem to be site specific [39]; tennis players have greater BMD in their dominant arm (impact site) compared to their non-dominant arm [40].

Dance training regiments during adolescence have been linked with low body weight, late onset of menarche and menstrual dysfunctions [41] which, in turn, increase the risk of developing low BMD and osteoporosis in later life [42]. The American College of Sports Medicine (ACSM) [11] portrays low BMD as a constituent of the female athlete triad. According to the ACSM, the female triad is a syndrome that involves the presence of three components - low energy availability, menstrual disturbance and low BMD - that are often interrelated. Thus, the female triad is spectrum of conditions that begins with energy or nutrient restriction, which may lead to the development of hypothalamic amenorrhea, with subsequently negative impact on BMD. Participants in physical activities that emphasise an aesthetic build and low body weight have been identified as potentially at-risk for developing the syndrome [11]. Given that dancing is an artistic expression in which physical fitness and aesthetics are key elements of performance [13], dancers might also fall in the same category. Indeed, observational data have

suggested that intense dance training during the growing years, combined with low energy intake and low body weight, might cause menstrual dysfunctions which, subsequently, could negatively affect the skeletal system [43]. Keay and colleagues [25] revealed that amenorrheic dancers have low Z-scores at the lumbar spine compared with controls, but eumenorrheic dancers have high Z-scores at femoral neck compared with normal population. Other published data demonstrated that as high as 40% of professional dancers could show symptoms of the triad [18]. Moreover, professional ballet dancers have been consistently found with low BMD [19,44–46]. All these authors agree that dancers are susceptible to menstrual disorders, and the weight-bearing exercise of dance training is unlikely to offset the harmful effects of amenorrhea/oligomenorrhea on BMD. The International Association of Dance Medicine and Science (IADMS) [17] published a statement highlighting BMD as a topic of major concern, associated with several health risks in dancers.

Contrary to the above, some authors advocate that professional dancers have higher BMD compared to controls, despite low body mass and late menarche [47]. Similarly, retired professional ballerinas [48] and vocational female dancers [27] were found not to be at risk of developing low BMD or osteoporosis. Consequently, the question of whether dancers are at risk of developing low BMD or not appears to be unanswered. Therefore, the aim of the present literature review was to systematically investigate and examine the information available in relation to prevalence and incidence of low BMD in female dancers.

METHODS

Literature Search and Identification

A systematic search of literature was undertaken using four electronic databases (Web of Science, PubMed, EBSCO, and Scopus). The search was extended to two specific dance science publications [Journal of Dance Medicine and Science (JDMS) and Medical Problems of Performing Artists (MPPA)] to ensure that we consider all relevant data. Material from the year of their inception up to January 2014, was identified using the terms “dance” and “ballet” combined with “BMD”, “bone density”, “osteoporosis” and “female athlete triad syndrome”.

It was included all studies involving bone measurement (at any site, with any kind of device) in dancers (any type of dance and competency level). Due to the limited number of randomised controlled trials (RCT), cross-sectional, non-randomised

longitudinal and retrospective cohort studies were also included. In contrast, editorials, conference proceedings, review papers, opinion papers, chapters in books, narrative papers and non-English language publications were excluded as they are generally considered of low quality studies [49]. It was also excluded studies that examined only male dancers because only two such papers were found. In contrast, studies that reported male and female data separately were included, but we only considered and analysed data on female dancers.

Papers on the prevalence of low BMD and associated factors, incidence of low BMD and risk factors, and treatment/prevention of low BMD were classified as “relevant material”. Papers with no such information were classified as “other studies”. This categorisation was assisted by two independent experts who appraised the relevance of each identified study. Prevalence and incidence were defined as the total number of existing cases with low BMD, and as the number of new cases with low BMD emerging during a specific period of time, respectively.

Article Quality Assessment

The quality of the eligible papers was assessed according to the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) system, an appropriate tool for assessing the quality of published reports [50]. RCT studies were assessed based on six parameters: (i) risk of bias, (ii) indirectness, (iii) imprecision, (iv) publication of bias, (v) large effect, and (vi) dose response. Non-RCT studies were evaluated using the following three parameters: (i) large effect, (ii) dose response, and (iii) all plausible residual confounding. GRADE classifies published material as high, moderate, low or very low quality, whereas RCTs start at a high quality level and non-RCT studies at a low quality level. Based on the information provided by the authors in each selected paper and applying GRADE’s parameters, two experienced appraisers rated them as “high” or “low” (no point given), “low plus one” (one point given) and “low plus two” (two points given). Was also considered the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [51].

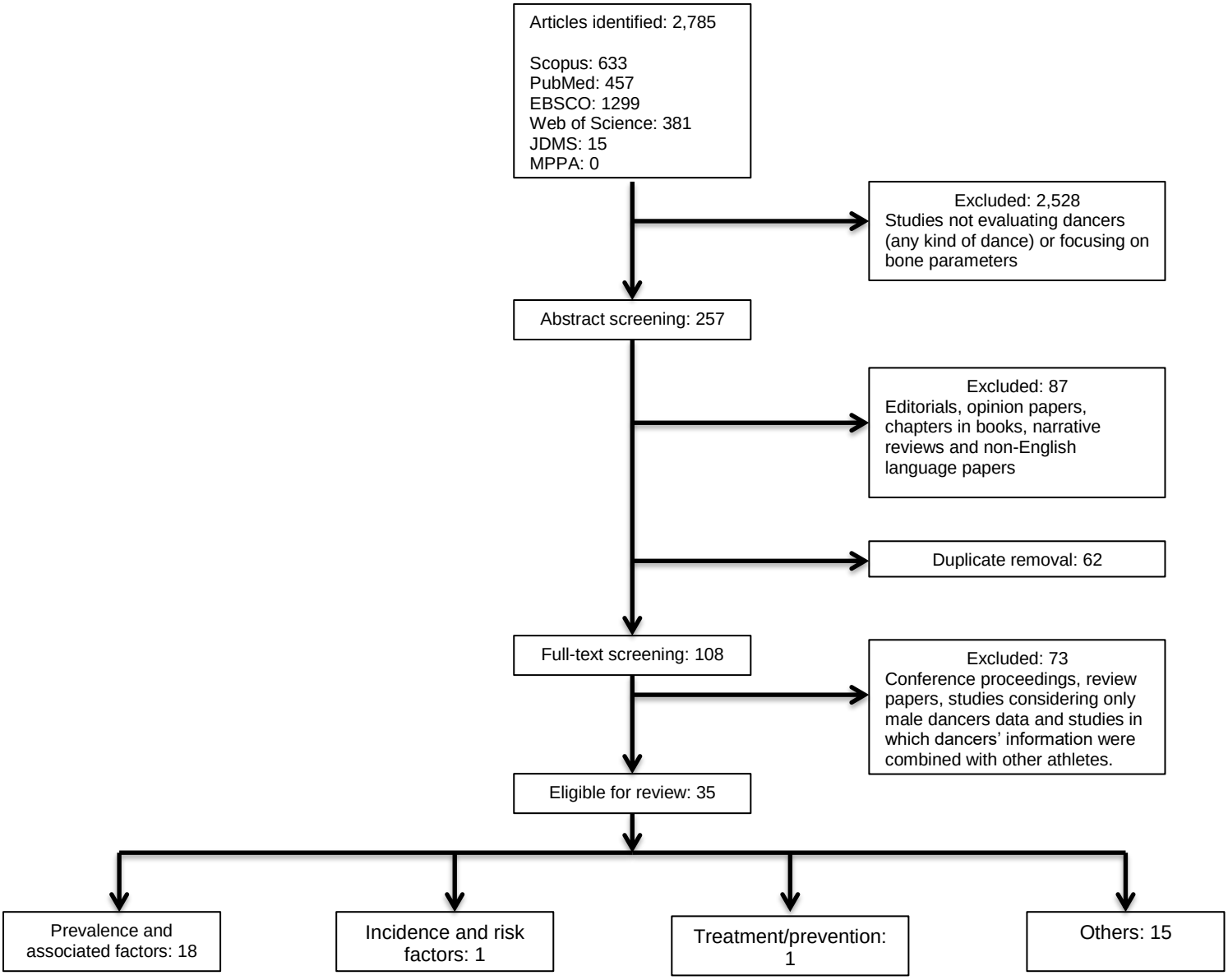


Figure. 1 Flow-chart of the identified and selected studies.

RESULTS

Using the terms "dance" and "ballet" combined with "BMD", "bone density", "osteoporosis" and "female athlete triad syndrome", 2785 outputs were initially emerged. After titles and abstracts were screened, 108 articles were identified as potentially relevant and were retrieved as full-texts. Following detailed examination, 73 of these articles were excluded, while only 35 fulfilled the set criteria (Figure 1). The latter 35 articles consisted of 31 cross-sectional studies, one longitudinal study, one mixed-longitudinal study, one retrospective study, and one RCT.

Of these 35 selected studies, 18 were identified as related to the prevalence of low BMD and associated factors, one to the incidence of low BMD and risk factors, one to treatment or prevention of low BMD, and 15 were classified as “other studies”.

Description of the Selected Studies

The quality scores of the 35 selected studies appear in Table 1. Twenty out of the 35 publications received a “low” score, 11 had a score of “low plus one”, three collected a “low plus two”, and only one was considered to be high quality (RCT).

Figure 2 shows the general characteristics of the selected papers. Of the 35 studies, 16 studies examined professional female dancers (three of which dealt with retired dancers), seven vocational dance students (age ranging from 16.4 to 20 years old), while 12 have studied non-professional dancers. Control groups were included in 27 studies.

Dual-energy X-ray absorptiometry (DXA) was the most frequently used methodology to evaluate bone parameters (24 studies). Of these, 13 evaluated both dance specific impact and non-impact sites, 16 reported diet analysis via a 3-day record, 12 examined hormone levels (most hormones relating to the menstrual cycle), and three studies assessed energy expenditure of professional dancers.

The main outcomes of the 27 studies that compared dancers’ bone mass with controls or normative values vary considerably with 15 of them revealing that dancers have low BMD at least in one site, eight suggest that dancers’ BMD is equal to non-dancers, and four studies disclosed that dancers have high BMD values (figure 3). However, it should be stressed that these outcomes come from published material classified as low quality based on GRADE system.

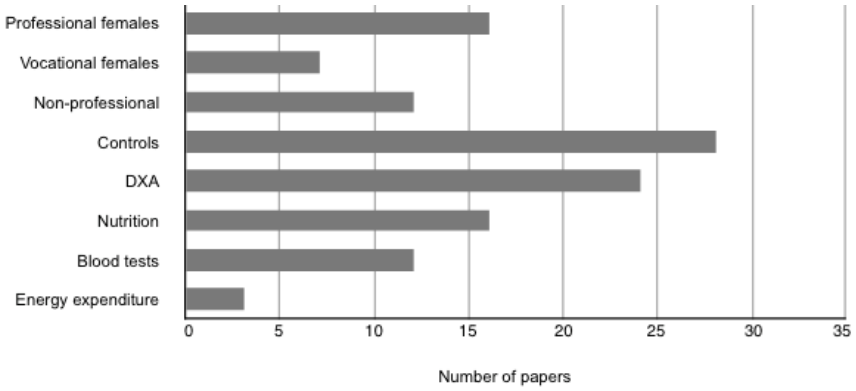


Figure. 2 General characteristics of the selected papers.

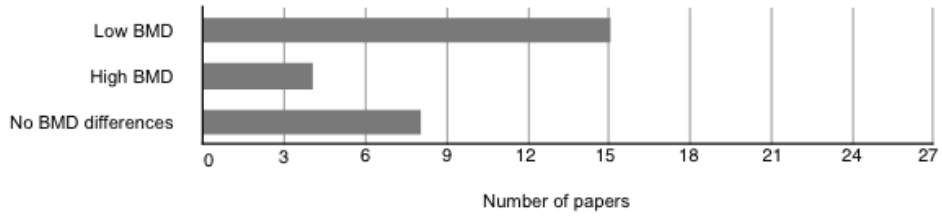


Figure. 3 Main outcomes of the 27 studies that compared dancers' bone mass with controls or normative values.

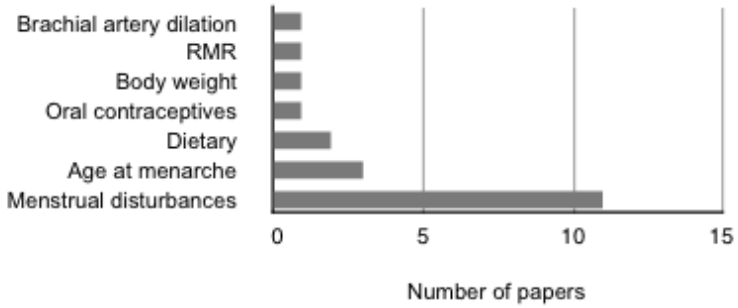


Figure. 4 Factors associated with low bone mineral density reported in the selected papers.

Prevalence of Low BMD and Associated Factors in Female Dancers

Eight cross-sectional studies fulfilled the eligible criteria on the prevalence of low BMD (Table 1). All studies were on ballet dancers, whose experience and level of performance ranged from vocational dance students to retired professional dancers. Variations were also found in terms of anatomical zones measured.

From the three studies that have examined professional female ballet dancers, one estimated the prevalence of low BMD at the lumbar spine (LS) to be 40% [44], while another one estimated the same parameter to be 23% [52]. The third study examined the presence of the female athlete triad syndrome in two professional ballet companies; it was found that 40% of the dancers exhibited symptoms of the triad, resulting in low BMD at the total body [18].

One study involving retired professional female ballet dancers revealed higher prevalence of osteoporosis at non-impact sites (26.7% vs. 15.8%), hip (6.9% vs. 3.9%), and femoral neck (FN) (17.8% vs. 16.8%) compared to controls, but lower prevalence of osteoporosis at the total body (8.9% versus 9.9%) and lumbar spine (11.9% vs. 15.8%) [48]. These authors also found that the prevalence of osteopenia in retired ballet dancers was 46.5% (39.6% for controls).

Female vocational dance students were investigated in some studies. Valentino and colleagues [43] reported that 60% of dance students and 55% of ex-dance students demonstrated a Z-score below 2.5 for lumbar spine, while 30 and 22% of dance and ex-dance students, respectively, exhibited a Z-score between one and two for the same site [according to the World Health Organization (WHO) [52], osteoporosis is considered when BMD value is at least -2.5 SD below mean for age, and osteopenia when BMD value is between -1.5 SD and -2.5 SD below mean for age]. Burckhardt and colleagues [19] estimated the prevalence of low BMD to be 37% at the LS in Asian and Caucasian female vocational dance students, whereas Chinese female dance students had significantly higher prevalence of osteopenia (26.7%) compared to age matched controls (14.3%) [26]. Regarding total body BMD in female vocational dance students, Yannakoulia and colleagues [16] found a prevalence of 37.8%.

Table 2 summarises all studies that provide evidence regarding prevalence of low BMD. It seems that vocational dance students had higher prevalence than their professional counterparts for the same sites. The average values for vocational dance students and professional dancers were: 47.7% vs. 25.9% at the lumbar spine, and 32.9% vs. 29.6% at total body. In professional female dancers, the highest prevalence is at non-impact sites (40%), followed by the total body (29.6%) and LS (25.9%). Compared with controls, both vocational dance students and professional dancers had a higher prevalence of low total body BMD. However, the prevalence of low BMD is lower in retired dancers at the LS and total body when compared to control populations of the same age.

Of all studies examining the prevalence of low BMD in dancers, seven reported certain associated factors (Table 1). This included taking oral contraceptives (those taking contraceptives had particularly low BMD) [44], menstrual disturbances [16,18,27,48], decreased brachial artery flow-mediated dilation [52], age at menarche [26], and dietary deficiencies [19]. Table 1 also depicts 10 cross-sectional studies that, although provided no information regarding prevalence, dealt with factors associated with low BMD in dancers. These factors included menstrual disturbances [29,45,46,53,54], dietary deficiencies [55,56], age at menarche [25,47], decreased body weight [28], and decreased resting metabolic rate (RMR) [55]. Figure 4 summarises these associated factors. It becomes clear that the most quoted factor for low BMD is menstrual disturbances. However, only seven studies used multivariate analyses to adjust for potential covariates [16,19,26,28,29,46,48], therefore these associated factors constitute

only preliminary evidence, as most of the relevant studies were observational in nature and used small sample sizes.

Incidence of Low BMD and Associated Risk Factors in Female Dancers

We found no data on incidence of low BMD in dancers. However, one study did provide information on potential risks factors (Table 1), demonstrating that female professional ballet dancers and vocational dance students with amenorrhea had low BMD at the LS compared to eumenorrheic dancers. These authors also reported that dancers who resumed menses significantly increased BMD at the wrist and LS (17%), but could not achieve normal levels. Nonetheless, this was classified as a low quality study due to a small sample size and low-level statistical analyses.

Treatment or Prevention of low BMD in Female Dancers

Only one study received high quality rating in this area (Table 1). This study adopted a placebo-controlled, randomised design to investigate the ability of oestrogen therapy to stimulate normalisation of bone mass in amenorrheic dancers [57]. Results indicated no significant difference between the treatment and placebo groups.

Other Studies

Although these 15 studies provided no direct information on prevalence/associated factors, incidence/risks factors, or treatment of low BMD in dancers, they could be useful as they included measurements of dancers´ bone mass (Table 1). These were all low quality studies, and most of them included other populations besides dancers [58–63]. Interestingly, published data obtained exclusively from dancers demonstrated conflicting results. Some indicated that bone mass of professional dancers and full-time dance students was significantly higher than controls [27,59] others did not [64], while some data disclosed similar values for dancers and non-dancers [65,66]. Table 1 also includes two studies, a cross-sectional [15] and a longitudinal [67], that have showed the positive effects of dance on bone using non-elite dance students. A cross-sectional study revealed that plasma leptin concentrations in adolescent female dancers is significantly lower in comparison to female controls, however, it is not a direct determinant of BMD in adolescent dancers [68]. Lastly, a study using quantitative ultrasound found that BMD measurements were significantly higher in dancers than in controls [69].

Table 1. Studies included in the systematic review and their main findings [a]

Group	Article	Participants	Main findings [b]	Quality
Prevalence and associated factors	Armann et al. [41] (1990)	Female prof (n=5, 26±4.6yr); controls (n=6, 16.2+1.2yr)	Prevalence: dancers over 20 years old (spine): 40%; dancers over 20 years old (radius): 40%. Associated factors: taking oral contraceptives.	Low
	Warren et al. [43] (1991)	Female prof (n=51, 13-29yr); controls (n=47, 13-29yr)	Prevalence: unknown. Associated factors: menstrual disturbances	Low (plus one)
	Karlsson et al. [26] (1992)	Female (n=25, 19-68yr); controls (n=42, age matched)	Prevalence: unknown Associated factors: menstrual disturbances	Low (plus one)
	Bass et al. [51] (1994)	Female prof (n=32); controls (n=23, age matched)	Prevalence: unknown Associated factors: menstrual disturbances	Low
	Young et al. [25] (1994)	Female vocational (n=44, 17±0.2yr); sedentary amenorrheic (n=18, 18.1±0.4yr); normal menstrual (n=23, 16.7±0.3yr)	Prevalence: unknown Associated factors: low body weight	Low (plus one)
	Lichtenbelt et al. [45] (1995)	Female prof (n=24, 22.6±4.5yr)	Prevalence: unknown Associated factors: age at menarche	Low
	Khan et al. [46] (1996)	Retired prof female (n=101, 51.1±1.4yr); controls (n=101, age matched)	Prevalence: osteoporosis: TB, 8.9 % dancers vs. 9.9% controls, with the corresponding values as follows: radius 26.7% vs.15.8%, hip 6.9% vs. 3.9%, FN 17.8% vs.16.8%, intertrochanteric and trochanter vs. 14.8%, LS 11.9% vs15.8%, any site 23.8% vs. 38.6%; osteopenia: any site, 46.5% vs.39.6%. Associated factors: menstrual disturbances	Low (plus two)
	Pearce et al. [42] (1996)	Female vocational students (n=41, 17.2±0.2yr); controls (n=46, 17.5±0.2yr)	Prevalence: unknown Associated factors: menstrual disturbances	Low (plus one)
	Keay et al. [22] (1997)	Retired prof female (n=57, 25-50yr)	Prevalence: unknown Associated factors: age at menarche; menstrual disturbances	Low (plus two)
	Valentino et al. [40] (2001)	Female vocational students (n=48, 21.5±3.7yr); ex-students (n=50, 22.3±1.8yr); controls (n=76, 22.5±1.5yr)	Prevalence: 60% of the dancers and 55.6% of ex-dancers had a Z-score below 2.5 at LS; 30% of dancers and 22.2% of ex-dancers had a Z-score between 1 and 2; controls: unknown. Associated factors: unknown	Low
	Kaufman et al. [53] (2002)	Female prof (n=21, 23.2±2.8yr); controls (n=27, 24.5±2.6yr)	Prevalence: unknown Associated factors: rest metabolic rate; low energy intake	Low
	Quintas et al. [54] (2003)	Female (n=33; 16.2±2.0yr); controls (n=90, 16.7±1.0yr)	Prevalence: unknown Associated factors: low body weight; low energy intake	Low
	Yannakoulia et al. [14] (2004)	Female vocational students (n=37, 20.7±1.8 yr)	Prevalence: 37.8% of dancers had lower total BMD Associated factors: menstrual disturbances	Low (plus one)
	To et al. [52] (2005)	Female vocational students (n=35, 17-19 yr); controls (n=35, 17-19yr)	Prevalence: unknown Associated factors: menstrual disturbances	Low
	Yang et al. [24] (2010)	Female adolescent (n=60, 16.5±0.7yr); controls (n=77, 16.4±0.6yr)	Prevalence: 26.7% of dancers found as having osteopenia compared with 14.3% for controls Associated factors: menstrual disturbances; age at menarche	Low
	Dolyle-Lucas et al. [15] (2010)	Female prof (n=15, 24.2±1.3yr); controls (n=24, 23.7±0.9yr)	Prevalence: 40% of dancers exhibited symptoms of the three conditions comprising the female a triad; controls: 0%. Associated factors: menstrual disturbances	Low (plus one)
	Hoch et al. [50] (2011)	Female prof (n=22, 23.2±4.7yr)	Prevalence: 23% had low BMD in 1 or more sites; 23% had low BMD at LS and 9% at TB; Z-scor were not met by any of dancers. Associated factors: low brachial artery flow-mediated dilation	Low
	Burckhardt et al. [16] (2011)	Female vocational students (n=127, 16.7±0.8)	Prevalence: 37% of dancers had LS BMAD below the fifth percentile. Associated factors: non-dietary protein intake	Low (plus one)

Table 1. Continued

Group	Article	Participants	Main findings [b]	Quality
Incidence and risk factors	Warren et al. [70] (2002)	2-year follow-up. Female prof and students from regional and national schools (n=54); controls (n=44) (22.4±4.6yr)	Incidence: unknown Risk factors: menstrual disturbances	Low
Treatment/ prevention	Warren et al. [55] (2003)	2-year follow-up. Amenorrheic (n=24) and eumenorrheic dancers (n=31) from regional schools and companies (22.0±4.6yr)	Intervention: Amenorrheic dancers receive placebo or Premarin, 0.625 mg for 25 days monthly, Provera, 10 mg, for 10 of these 25 days (hormone therapy) for 2 years. Outcomes: No difference in BMD between treated or placebo group	High
Other studies	Wolman et al. [56] (1991)	Female prof (n=10, 20.7-25yr); runners; rowers; controls (n=13, 26.5-30.3)	Prevalence: unknown Associated factors: unknown Dancers had similar BMD values compared with controls	Low
	Frederick et al. [58] (1992)	College dancers (n=14, 17-25yr); postmenopausal women; track team; controls (n=14, 17-25yr)	Prevalence/Associated factors: unknown No significant differences in BMD among the four groups	Low
	Foldes et al. [63] (1997)	Female high-school dance students (n=27, 15.6±1.2yr); controls (n=27, 15.6±0.8yr)	Prevalence: unknown Associated factors: unknown BMD did not differ between groups	Low (plus one)
	Cuesta et al. [62] (1996)	Female (n=15, 25.1±3.8yr); controls sex- and age-matched	Prevalence: unknown Associated factors: unknown BMC low in arms when compared to controls (both female and male)	Low
	Khan et al. [71] (1998)	Retired female prof (n=101, 51.4±14.3yr); controls (n=99, n=51.5±16.0yr)	Prevalence: unknown Associated factors: unknown Hours of ballet training per week during infancy was positively associated with BMD	Low (plus two)
	Bennell et al. [13] (2000)	Non-elite female students (n=78, 9.6±0.8yr); controls (n=63, 9.6±0.8yr)	Prevalence: unknown Associated factors: unknown BMC upper limb lower in dancers; BMD high in dancers at FN, hip; no differences at LS.	Low
	Tsai et al. [64] (2001)	Female (n=29, 16.3±0.5yr); controls (n=20, 16.6±0.8yr)	Prevalence: unknown Associated factors: unknown Similar BMD at LS and FN between groups	Low
	Munoz et al. [59] (2004)	Female dancers (n=12, 16.2±2.0yr); rhythmic gymnasts; controls (n=14, 16.9±1.0yr)	Prevalence: unknown Associated factors: unknown BMD at LS normal in all groups; significant decrease found in dancers and gymnasts at forearm compared with controls	Low
	Matthews et al. [65] (2006)	Non-elite dancers (n=82, 8-11yr); controls (n=61, 8-11yr)	Prevalence: unknown Associated factors: unknown Dancing is associated with a positive effect on bone mass	Low (plus one)
	Oral et al. [67] (2006)	Female (n=26); controls: age- and sex-matched (n=100)	Prevalence: unknown Associated factors: unknown Dancers had significant higher calcaneal QUS measurements compared to controls	Low (plus one)
	Kilicarslan et al. [57] (2007)	Female dancers (n=22, 29.8±3.0yr); controls (n=20, 28.6±2.6yr	Prevalence: unknown Associated factors: unknown Z-scores at the LS and FN significantly greater in dancers; no significant difference in Z-scores at the forearm	Low

Table 1. Continued

Group	Article	Participants	Main findings [b]	Quality
	Yang et al. [66] (2009)	Female adolescent (n=60, 16.5±0.7yr); controls (n=77, 16.4±0.6yr)	Prevalence: unknown Associated factors: unknown Plasma leptin levels is not a direct determinant of BMD	Low
	Hinrichs et al. [60] (2010)	Female dancers (n=13); runners; team athletes; triathletes; combat players; controls (n=61)	Prevalence: unknown Associated factors: unknown BMD at LS was the lowest in dancers.	Low
	Friesen et al. [61] (2011)	Female dancers from university (n=32, 22.1±1.4yr); controls (n=30, 21.4±1.5yr)	Prevalence: unknown Associated factors: unknown BMD did not differ between groups; BMD at LS and hip higher in dancers.	Low
	To et al. [23] (2011)	Vocational female students (n= 47, 17-20yr); controls (n=36, 17-20yr)	Prevalence: unknown Associated factors: unknown Dancers do not exhibit low BMD at any site	Low (plus one)

Prof = professional; BMD = bone mineral density; BMC= bone mineral content; BMAD = bone mineral apparent density; QUS = quantitative ultrasound; LS = lumbar spine; FN = femoral neck; TB = total body

[a] Values are mean ± SD or range except where stated otherwise

[b] Prevalence/ Incidence = prevalence/ incidence of low BMD

Table 2. Prevalence estimates for low bone mineral density in female dancers (data manually calculated by the present authors from all published papers included in the "prevalence and associated factors" group)

Prevalence						
	Vocational		Professional		Retired professional	
	Dancers	Controls	Dancers	Controls	Dancers	Controls
Femoral neck	No published data	No published data	No published data	No published data	17.8%	16.8%
Lumbar spine	47.7%	No published data	25.9%	No published data	11.9%	15.8%
Non-impact sites	No published data	No published data	40%	No published data	26.7%	15.8%
Total body	32.9%	14.3%	29.6%	0%	8.9%	9.9%

DISCUSSION

Unlike athletic populations [72–74], there has been no published information on the short or long-term health consequences of low BMD in professional and vocational dancers. Therefore, the aim of the current review was to systematically examine the available information regarding prevalence of low BMD in this population.

To my knowledge, this is the first systematic review on dancers' BMD. It was found that the reported data are ambiguous and limited to principally observational studies of average to low quality. Specifically, only eight out of the 35 finally selected studies dealt with prevalence of low BMD in dancers, 17 on associated factors, one on risk factors, one on treatment and none reported on incidence of low BMD. The majority of the studies have focused on the assessment of professional female ballet dancers, and only three published reports provide prevalence estimates for control populations [18,26,48]. Therefore, it is difficult to draw firm conclusions as to whether dancers have higher or lower BMD prevalence compared to the general population since there is no published information about prevalence of low BMD in control populations at the FN, LS and non-impact sites in both students and professional dancers. In addition, data shown in table 2 regarding prevalence estimates for student controls at total body were provided by a single study [18]. Similarly, all prevalence estimates for retired professional dancers (and respective controls) shown in table 2 also came from a single study [48], as well as prevalence estimates for professional controls [26]. Therefore, there is a need to confirm these values in future high-quality and well-designed research studies.

At least one study, using quantitative computed tomography, showed that 60% of vocational dance students and 55% of ex-dance students demonstrated a Z-score below 2.5 for LS, and 30 and 22% of dance and ex-dance students exhibited a Z-score between one and two for the same site [43]. However, it is worth noting that these authors used the WHO criteria to diagnose osteoporosis/osteopenia, which are only suitable for DXA measurements, not for quantitative computed tomography [75]. Furthermore, WHO criteria were designed for postmenopausal women, not for young and active individuals. Thus, the ACSM suggests adherence to guidelines from the International Society of Clinical Densitometry (ISCD) instead of guidelines from the WHO when considering athletic populations [11].

It has been noticed that there is no information on the prevalence of low BMD in vocational dance students at non-impact sites and FN (impact site). However,

given the nature of dance training and the importance of the growing years for bone mass development [75,76], it could be sensible for future studies to assess impact and non-impact sites in vocational dance students. Further, there are no studies reporting incidence estimates of low BMD in dancers. To address this issue longitudinal designs are needed; 31 out of the 35 finally selected studies for the current review adopted cross-sectional designs.

Out of the 17 studies included in this review that provided information on factors associated with low BMD, 11 studies reported menstrual disturbances as associated factor and one study as a risk factor. However, these associations should be treated with caution given that all studies used a small sample size, and the majority of them did not applied multivariable analyses to adjust for potential covariates and were, therefore, classified as low quality based on the GRADE system. Moreover, research involving associated factors has been limited by the fact that they restrict analyses mainly to menstrual disturbances and nutrition. There are other factors that may potentially play a significant role on bone metabolism, such as bone mass related hormones [77]. Future studies should incorporate possible associated and risk factors within a multivariate design.

There have also been limited investigations on the effectiveness of different interventions within a RCT design; only one study used such a design [57]. Therefore, current findings on this issue can only be treated as preliminary evidence that need to be confirmed in appropriately designed studies. Of particular interest is that some of the existing literature on dancers´ BMD suggest that bone mass may not accumulate in the same manner in adolescents as in the mature individuals, since a delay in menarche may affect bone mass gains [46]. To date, there is no evidence supporting this claim as only one study used a mixed longitudinal design [67]. However, these authors examined female non-elite dancers and, therefore, their findings are not transferable to elite vocational dance school populations.

The conflicting results found herein (i.e., studies showing lower BMD in dancers and others showing higher BMD) could be due to differences in dancers' performance levels, study design and methodologies employed. DXA has been the most used device adopted by the studies of this review, confirming that it is the best current test to determine bone mass [78]. However, the anatomical sites measured and the sample characteristics of these studies differ, a fact that might have implications on BMD outcomes. BMD might be quite diverse in subjects with different

training levels [79], ages [80], and ethnicities [81]. Indeed, aging itself is considered to be a risk factor for low BMD and osteoporosis. While childhood and adolescence are crucial periods for bone mass gains, adulthood is considered to be a bone-mass-maintenance period; during older adulthood, rapid bone loss can occur [77]. It is expected, therefore, that the prevalence of low BMD in dancers will change according to growth stage and age. Nonetheless, none of the included studies reported prevalence of low BMD in dancers according to age. In fact, only three studies of the "prevalence and associated factors" group considered age cluster – adolescence [19,26] and older adulthood [48].

Although scientific research has not established with certainly the intensity, frequency and volume of exercise that will increase BMD in the general population and athletes, published reports suggest that as few as 2-3 training days per week of combined weight-bearing exercises with high-impact exercises (e.g., jumping) are sufficient to stimulate bone metabolism [10]. In general, dancers are involved in daily classes and several hours of rehearsing [82] of medium physical demands [14], whereas muscular strength and jumping play a key role for performance [83]. However, although dancing has been hailed as an osteogenic activity [48], we found no studies reporting on the thresholds (intensity, frequency, volume) above which dancing might stimulate bone mass gains. Furthermore, none of the included studies refer to the possible relationships between dance training loadings (intensity, frequency, volume), menstrual disturbances and bone mass acquisition. In contrast, it has been suggested that professional dancers are exposed to high risk of injury [84–86], but interestingly no studies have been identified for reporting a possible association between low BMD and dance injuries. Finally, there are no available data on incidence of low BMD in dancers. Therefore, trends over time cannot be analysed and risk factors cannot be clearly determined.

CONCLUSIONS

The present systematic review cannot answer the fundamental question as to whether there is a high prevalence and incidence of low BMD in professional and vocational female dancers. Future research needs to focus on high quality research designs that allow associated and risk factors to be examined within a controlled environment. Future research should also distinguish between dancers´ training levels, ages and ethnicity.

CHAPTER 3: RATIONALE AND AIMS OF THE THESIS

The previous Chapter (Chapter 2) allowed the identification of literature gaps, strengths and limitations regarding dancers' bone health. This first task provided me some evidence to delineate the type of research to be conducted for this PhD. The following gaps have been identified:

Employed methodology

It was clear that the main findings regarding dancers' bone health come from studies classified as low quality based on the GRADE system. There is also a clear gap in terms of methodology in assessing BMD, whereas, for instance, some papers used DXA to assess bone mass, and others used different devices. Further, current studies used the WHO criterion to diagnose young dancers with low BMD; however, this criterion has been designed only for postmenopausal women.

What this PhD adds: The current PhD tried to increase the research quality of its studies by following principles of the Equator Network Guidelines. DXA is used in all studies as it is the most suitable device for bone mass measurements [78]. For accuracy, this PhD also considers the analysis of bone data according to guidelines from the ACSM and ISCD (not WHO criterion).

Participants

The sample size included in the published studies is small and comes from different expertise levels (i.e. non-professionals, professional, vocational students and retired); the use of controls is also limited. Further, it is clear the lack of data in relation to male dancers (only two studies focused on male dancers).

What this PhD adds: Studies conducted on a well-defined population of elite dancers. Female and male professional ballet dancers, and female and male vocational dance students have been studied (sex- and aged-matched controls also included).

Anatomical sites measured

BMD gains from exercise are site specific, which means that exercise affects differently the skeleton depending on the areas where the stimulus is applied [40,87]. Therefore, when studying exercise populations it is important to consider the specificity of the activity to determine which anatomical sites are relevant and which

sites should be considered for bone mass measurements. In dancing, the high levels of muscular strength for technical performance and the weight-bearing activity associated with jumping may stimulate bone-forming cells at the FN and LS, whereas (basically) no forces nor impacts are applied at the arms. Therefore, when studying dancers' bone health is important to consider both impact sites (FN and LS) and non-impact sites (forearm).

What this PhD adds: the current PhD thesis analysed (in all conducted studies) bone mass parameters at both impact (FN and LS) and non-impact sites (forearm).

Only cross-sectional data available in elite dance students

Current published data only considered the cross-sectional assessment of adolescent dancers (ranging from 16 to 18 years old), which do not allow the analysis of BMD trends over time, i.e. how vocational dance students acquire bone mass as they grow and as they progress in their professional training; there is no data on dancers' bone status during early adolescence. Therefore, longitudinal data is of relevance to investigate predictors of BMC and BMD changes throughout growth, a critical period for bone mass acquisition [3].

What this PhD adds: this PhD thesis included longitudinal data of female and male vocational dance students, whose age ranged from 10-18 years old, covering the entire years of vocational school.

Lack of consistency

Considering the main outcomes, the majority of published papers on the topic revealed that female dancers (both professional and vocational) have low BMD at least in one anatomical site, whereas others showed that dancers have similar or high BMD values compared to normative values or controls. In accordance, it is clear the ambiguity on the topic. Nevertheless, data on dancers' bone health status, both in female and male, is relevant because if dancers are currently at increased risk for low BMD, the number of cases and severity might increase after ceasing the profession. Further, vocational dance students may enter adulthood with low BMD, which may further impair the peak mass attainment, increasing the chances of precocious bone losses. All of these scenarios may affect dancers' wellbeing and quality of life. Thus, it is crucial to first clarify if professional dancers and vocational students represent part of the population at increased risk for low BMD.

What this PhD adds: this PhD thesis first addresses the fundamental question as weather professional and vocational dance students (both female and male) have increased odds of developing low BMD compared to normal population (non-exercising individuals, representative of the population). Further, this PhD thesis also includes original data on bone health status of vocational dancers prior to dance training stimuli.

Associated factors with BMD

Some gaps in the available literature might also exist regarding the factors associated with BMD in dancers. The majority of the studies did not adjust the data for potential covariates. Furthermore, probably due to issues related with the female athlete triad, current research mainly restricts the analyses to menstrual disturbances, body weight and nutrition. This is a limitation due to the fact that the aetiology of low BMD and osteoporosis is multifactorial; both genetic and environmental factors are key factors in determining bone mass phenotypes. Accurate information on factors associated with BMD in dancers is of relevance to identify those at risk for low BMD.

What this PhD adds: Original studies of the present PhD thesis aimed at conducting a multidisciplinary approach by investigating not only the association between BMD with traditional osteoporosis risk factors (i.e. menstrual disturbances, body mass and nutrition), but also with other factors that have not been considered previously in dancers such as genetics and growth hormonal profile. BMD data is adjusted for potential confounders in all studies of this thesis.

Lack of interventional studies

Only one study tested the effectiveness of an intervention to restore bone mass to normative values in professional dancers with low BMD [57]; this study found no differences between the treatment and placebo groups. This lack of effectiveness could be due to limitations inherent to the adopted methodology, but it can also be hypothesized that the lack of positive results could be due to the fact that the treatment was not appropriate for the aetiology of low BMD in the studied population.

What this PhD adds: Before adopting a RCT design, there is a need to first clarify the factors associated with BMD in professional ballet dancers and vocational dance students, as well as the potential mechanisms involved in the determining bone mass

phenotypes. By investigating these topics, the current PhD thesis could be used to inform future RCT about the number of participants to include in an intervention, as well as providing directions on the treatment to be implemented.

Considering all the aforementioned topics, **the aim of the present PhD thesis is a) to investigate bone health status of professional ballet dancers and vocational dance students; and b) to investigate associated factors and mechanisms involved in dancers' bone health.**

Specifically, this Thesis aims at:
Specific aim 1: Assessing levels of areal and volumetric measures of bone mass in first year female vocational dance students (selected for professional training) and aged- and sex-matched controls.

Specific aim 2: Assessing the prevalence of low BMD in female and male professional ballet dancers and vocational dance students (and aged- and sex-matched controls), and investigate its association with body weight, fat mass (FM), lean mass (LM), menarche and maturation.

Specific aim 3: Modelling BMC and BMD accruals in female vocational dance students and aged- and sex-matched controls, and determining whether circulating levels of oestrogens, GH and IGF-1 are significant predictors of BMC and BMD changes throughout growth in female vocational dance students.

Specific aim 4: Determining whether nutrition, energy expenditure, and energy availability are significant predictors of BMC and BMD changes throughout growth in female and male vocational dance students.

Specific aim 5: Assessing the association of SNP in the Wnt/β-catenin and ER signalling pathways with low BMD in female and male professional ballet dancers and vocational dance students (and aged- and sex-matched controls).

CHAPTER 4: METHODS USED IN THIS PhD

ETHICAL APPROVAL

This PhD study conformed to the standards set by the Declaration of Helsinki and was approved by the NHS Health Research Authority, United Kingdom (Proc.14/WM/0008 and 14/WM/0009), and by the ethics committee of the Regional Administration of Health of Lisbon, Portugal (Proc. 063/CES/INV/2012).

METHODS

Professional ballet dancers (6-8 hours a day of training) and vocational dance students (school that offers full-time dance training to become professional dancers; students have to audition for a place; 4-8 hours a day of training) have been recruited from Portugal and England. In total, two professional ballet companies (one in Portugal in England) and two vocational dance schools (one in Portugal and other in England) were invited to participate in the study. The specific methodology employed in all volunteers included bone mass measurements (main outcome), anthropometry, menstrual, biological maturation, nutrition, energy availability, hormonal analysis, physical exercise in terms of bone specific loading and genes genotype.

Participants' recruitment

Pilot studies were administrated in one professional ballet company and one vocational dance school in order to calculate the sample size needed for BMD assessment; aged- and sex-matched controls were also included in both cases. In a sample of 22 female professional ballet dancers (22 matched-controls) and 10 male professional ballet dancers (10 matched-controls), the prevalence of low BMD (*Z*-score of -1.0) at the LS was found to be 32% (vs. 5%) in female dancers and 20% (v. 0%) in male dancers. It was subsequently estimated that 42 female participants and 46 male participants in each group were needed to reach significance (90% power, α=0.05). Similarly, in a sample of 36 female vocational students and 36 matched-controls, low BMD (*Z*-score of <-2.0) at the LS was found in 36% and 6%, respectively. Based on this finding, it was estimated that 37 participants were needed in each group to obtain 90% power, with α=0.05. As previously mentioned, and assuming participants´ non-response and possible dropouts, two professional ballet companies and two vocational dance schools were approached.

An introductory letter describing briefly the study was sent to the executive boards of the ballet companies and vocational schools (using Portuguese language in Portugal and English in UK). Following boards' permission, professional dancers and vocational students (and respective guardians) were presented with the purposes of the study; 166 dance students (45.8%) and 96 professional dancers (68.6%) volunteered. All volunteers completed a questionnaire concerning their race, medical history, and past/current calcium/vitamin D supplementation. Given the differences in bone mass values between individuals from different races [81], only participants referring themselves as white European-Caucasian dancers were included. Eligible criteria also included participants with no illnesses or treatments that might affect bone metabolism, not taking medication known to influence bone metabolism and no calcium/vitamin D supplementation. Women taking oral contraceptives and hormonal therapy were excluded. Based on these criteria, the studied population included in this PhD consisted of 160 vocational dance students and 86 professional ballet dancers. Details of the recruited dance population and its participation rate is available in Figure 5.

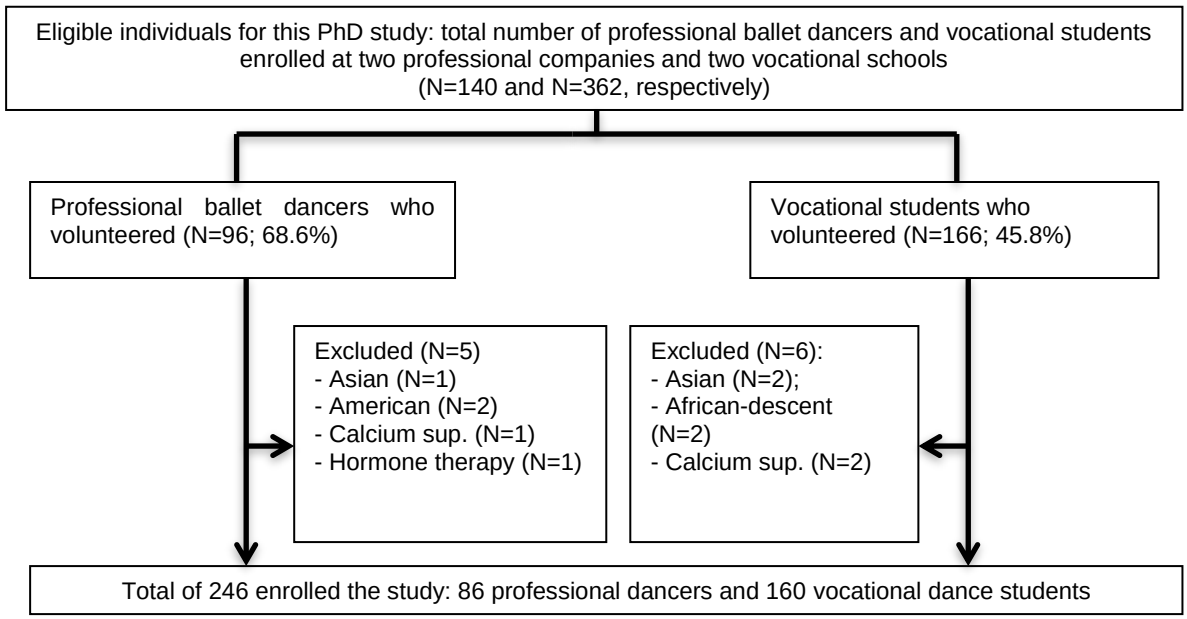


Figure 5. Dance population included in this PhD

Non-exercising participants were recruited from two local state schools and local Universities to act as controls (in Portugal). Eligibility criteria for controls were set according to dancers' characteristics, i.e. controls were only considered eligible if

they were of the same sex, age (defined as decimal age; 12-months difference of a dancer) and race (white European-Caucasian) as dancers. Exclusion criteria included those who participated or had previously participated in organised physical activities/sports outside school curriculum; children participants involved in physical education sessions at their school were not excluded. Control participation was also restricted to those who had received/were receiving medications known to affect bone metabolism and to who reported illnesses/treatments that might affect bone metabolism. Following consent from the respective boards of directors, the study was advertised by the school/ universities authorities. Out of the 282 responses, 167 fulfilled the current criteria and were included in the study (controls for vocational dance students: 97; controls for professional ballet dancers: 70).

Participants' measurements

In Portugal, cross-sectional data for professional dancers were collected between February 2013 - May 2013 (bone mass, anthropometry, menstruation, nutrition, energy availability and bloods), whereas longitudinal data were collected annually for three consecutive years in Portuguese vocational dance students, starting at January 2013 and finishing at March 2015. Annual collection occurred within the same period as the baseline measurements. Specifically, information on bone mass, anthropometry, menstruation, biological maturation [estimation of the age at peak height velocity (PHV) and Tanner stage], nutrition (energy intake, calcium, fat, carbohydrates) and energy availability were collect each January for vocational dance students, whereas the same information was collected each March in controls. Bloods were collected each January in both groups. Information on physical exercise in terms of bone specific loading was collected on the last year of measurements.

In Portugal, a total of 70 professional ballet dancers (70 aged- and sex-matched controls) and 101 vocational dance students were assessed at baseline (97 aged- and sex-matched controls). From 2013 to 2014, 12 vocational dance students were additionally recruited, while 12 female dancers and 5 male dancers withdraw the study due to professional dance training dropout or illness. Considering Portuguese controls, 9 female controls and 7 male controls withdrew the study due to family relocation or loss of interest. From 2014 to 2015, an additional 12 male dance students, six controls for female dance students and 11 controls for male dance students withdrew the study for the reasons previously mentioned (in Portugal).

In UK, cross-sectional data for professional dancers were collected between June 2013 - September 2013 (bone mass, anthropometry, menstruation, nutrition, energy availability and bloods), whereas longitudinal data were collected annually for two consecutive years in vocational dance students, starting at May 2013 and finishing at May 2014. Annual collection occurred within the same period as the baseline measurements; all measurements (the same information collected in Portugal) in British vocational dance students was collected during May (May 2013 and May 2014). A total of 16 professional ballet dancers and 59 vocational dance students were initially measured at baseline. From 2013 to 2014, 16 British vocational dance students withdraw the study due to professional dance training dropout or illness. Controls for UK were only recruited to match (aged- and sex-matched) with dancers for study 2 and study 5; a total of 90 controls were assessed between 2013-2014.

Anthropometry, menstrual, nutritional intake and energy availability

Chronological age was obtained as decimal age (date of birth minus measurement date). Height, sitting height and body mass were measured in t-shirt, shorts and bare feet using a stadiometer (Seca, Seca217 portable stadiometer, Hamburg, Germany) with accuracy of 0.1 cm and an electric scale (TANITA BC-418 MA Segmental Body Composition Analyser; Tanita Corporation, Tokyo, Japan) with an accuracy of 0.1 kg. All measurements were administered by the same investigator twice (mean of the two measurements were recorded).

All participants were presented with a questionnaire to determine age at menarche and regularity of menstrual cycles. Amenorrhea was defined as the absence of menses for three consecutive months, whereas oligomenorrhea was considered when menstrual cycles occurred at intervals of greater than 35 days.

Pubertal development was assessed using Tanner staging (breast and public hair stage in girls; genitalia and pubic hair stage in boys) by self-reporting. Standard line drawings and written descriptions were provided, and participants selected the picture that most accurately reflected their appearance [88]. Biological maturity was assessed using the offset equation [89]. Based on this equation, the year(s) to/from peak height velocity (PHV) and an estimation of the age at PHV were predicted in all participants at one-year interval.

Nutrient intakes were recorded via a 3-day food diary, previously validated [90]. Participants were asked to record all food and beverages consumed during two school days and one weekend day following appropriate instructions. The software Food Processor SQL Edition, version 9.8.1. was used. During the same week that nutrition information was collected, energy expenditure was also estimated using an accelerometer – SenseWear [91]; each participant used the device for 7 consecutive days. Energy availability was further estimated using standard protocols (http://www.femaleathletetriad.org/calculators/); information on dietary energy intake (provided by the food diary), exercise energy expenditure (information retrieved from the accelerometer), and body fat percentage (measured by DXA) was used for the estimation of energy availability.

Hormonal analyses

Blood samples were collected in early morning after an 8-hour fasting. In menstruating subjects, samples were collected during the follicular phase of the menstrual cycle (fifth and ten days after the onset of menstrual bleeding). Plasma oestrogens concentrations were measured by electrochemiluminescence immunoassay (ECLIA) kit (06656021190 Estradiol G3 Elecsys cobas and 100, Roche Diagnostic Systems); the intra-assay and inter-assay CV's were below or equal to 2.4% and 2.7%, respectively. Serum GH concentrations were measured by an immunoradiometric assay kit (IRMA GH, ref. IM1397) from IMMUNOTECH SAS, (Prague, Czech Republic); the intra-assay and inter-assay CV's were below or equal to 2.7% and 7.1%, respectively.Serum IGF-1 concentrations were measured by an immunoradiometric assay kit (IRMA IGF-I, ref. A15729) from IMMUNOTECH SAS, (Marseille, France). The intra-assay and inter-assay CV's were below or equal to 6.3% and 6.8%, respectively. Serum sclerostin concentrations were measured by an ELISA assay kit (Human SOST/Sclerostin Quantikine ELISA Kit, Ref DSST00), from R&D Systems, Inc. (Minneaplolis, MN 55413, USA). The intra-assay and inter-assay CV's ranged between 1.8-2.1% and 8.2-10.8%, respectively. Blood samples were submitted to centrifugation at 2500g for 10 min; serum samples were stored at -80ºC until they were analysed.

Physical exercise in terms of bone specific loading

The bone-specific physical activity questionnaire (BPAQ) was administrated to all participants included in the longitudinal analysis (data collected on the last year of measurements – 2015 in Portugal and 2014 in UK). Participants were asked to record all regular physical exercise activities performed throughout their life, including years and frequency (times/week) of participation. A score for physical exercise in terms of bone specific loading was further derived for each participant. The BPAQ has been described in detail previous [92]. Briefly, the BPAQ has been designed to capture bone-relevant weight-bearing exercise history (an algorithm was created to account the factors of load intensity, years of participation, and frequency of current and historical physical exercise) [92]. It has been reported that the BPAQ score is positively associated with DXA BMD outcomes [92]. The BPAQ was originally validated for young adults, however, it can also be applied to children (a specific age weighting factor in the algorithm is applied) [92]. In accordance, several reports have been using BPAQ in children and adolescents to predict bone-relevant weight-bearing exercise [93–95]. Control participants have not been involved in methodical exercise (exclusion criteria included those who participated or had previously participated in methodical physical exercise activities outside school curriculum). In order to account for the physical education lessons at school (twice a week) it was assumed the following activities for control participants: walking/hiking, running/jogging, soccer and jump rope.

Genes and SNP selection

Genes of the Wnt/β-catenin and ER signalling pathways with potential biological function, and further established in genome-wide association studies (GWAS) and meta-analysis as genes related to low bone mass phenotypes, were identified according to literature reports. This resulted in the identification of four major genes: *SOST*, *LRP5* (Wnt/β-catenin pathway), *ESR1* and *ESR2* (ER signalling pathway). SNPs in or near these genes reported to have a significant association with BMD variation and risk of osteoporosis in European populations were identified using genetic variation databases such as Hapmap and NCBI, and previous association studies on European populations. The following SNPs were identified in *SOST*: rs851054, rs851056, rs10534024, rs4792909, rs9902563; *LRP5*: rs3736228,

rs2306862, rs682429, rs491347, rs3781590, rs2508836, rs643892, rs312786; *ESR1*: rs2234693, rs9340799; *ESR2*: rs1256030, rs960070.

Characteristics of each SNP were examined using the Ensembl database, and linkage disequilibrium (LD) analyses were performed using Haploview 4.1 with data available on HapMap. Within *SOST* region, rs4792909 and rs9902563 were located 32275 and 74118 bases, respectively, far downstream of the *SOST* start site. The SNPs rs851054 and rs851056 were in complete LD (R^2=1), while rs10534025 were not in LD with any of the other SNPs. Both rs3781590 and rs643892 in *LRP5* were in complete LD (R^2=1) with another SNP that has not been previously studied in relation to bone mass phenotypes (rs587808), whereas rs2508836, rs491347 and rs312786 were not in LD with any of the others SNPs. Information on rs682429 was not available on HapMap by the time of the LD assessment. The minor allele frequency (MAF) of rs3736228 and rs2306862 was low within the European ancestry population (CEU) (0.12 and 0.13, respectively). Considering the SNPs in *ESR1*, LD analyses revealed that rs2234693 and rs9340799 were in moderate LD (R^2=0.6), whereas rs1256030 and rs960070 in *ESR2* revealed high LD (R^2=0.9). Based on the attributes described, the following eleven SNPs were selected for genotyping: *SOST*: rs851054, rs10534024; *LRP5*: rs682429, rs491347, rs2508836, rs587808, rs312786; *ESR1*: rs2234693, rs9340799; *ESR2*: rs1256030, rs960070.

Genotyping

Genomic DNA was isolated from blood using the MagNA Pure LC DNA isolation kit (Roche, Switzerland) according to product specifications. Primers were generated from the genomic sequence using Primer-BLAST and its specificity determined using BLASTn. DNA was amplified with the QIAGEN Multiplex PCR Kit (Qiagen, Germany), either in single PCR reactions (SNP rs312786) or in two sets of multiplex reactions (set 1: SNPs rs2234693, rs960070, rs682429, rs587808 and rs851054; set 2: SNPs rs9340799, rs1256030, rs491347, rs2508836 and rs10534024). PCR products were purified using Sephadex G-50 fine (Sigma-Aldrich, USA) columns on a filtration plate and genotypes determined using the Genetic Analyzer 3130 and 3130xl (Applied Biosystems).

Bone mass assessments

BMD (g/cm^2) and (BMC) (g) were determined for non-dominant forearm (33% radius), LS (L1-L4) and FN. Body composition was assessed through a DXA whole-body scan [FM and LM (Kg)]. To estimate volumetric density, bone mineral apparent density (BMAD) (g/cm^3) was calculated for all sites using previously described formulas [96]. Participants were assessed in different centres using DXA GE Lunar Prodigy and Hologic Discovery Wi. Although previous studies have demonstrated a high correlation between Lunar and Hologic DXA BMD measurements [97–99], there is a tendency for Lunar model to inflate BMD values by 15% compared to Hologic [100]. Therefore, in addition to the daily calibration required from each DXA manufacturer, cross-calibration of the two scanners was conducted using a group of 20 independent participants. These participants were measured with both Lunar and Hologic within a period of 5 days. Regression equations using BMD from Lunar as dependent variable and BMD from Hologic as independent variable were performed from the data obtained from the participants included from cross-calibration. The correlation between the two DXA models were high (forearm BMD: r=0.96, adjusted r^2=0.93, std. error of estimate=0.03; LS BMD: r=0.96, adjusted r^2=0.92, std. error of estimate=0.05; FN BMD: r=0.97, adjusted r^2=0.93, std. error of estimate=0.05; forearm BMC: r=0.98, adjusted r^2=0.96, std. error of estimate=0.09; LS BMC: r=0.96, adjusted r^2=0.92, std. error of estimate=4.41; FN BMC: r=0.94, adjusted r^2=0.88, std. error of estimate=0.46; bone area (BA) forearm: r=0.82, adjusted r^2=0.66, std. error of estimate=0.16; LS BA: r=0.88, adjusted r^2=0.76, std. error of estimate=4.64; BA FN: r=0.87, adjusted r^2=0.75, std. error of estimate=0.35). The Hologic BMD, BMC and BA data were further converted to the Lunar data using the following equations: forearm BMD Lunar = -0,085263 + 1,356535*Hologic; LS BMD Lunar = 0,030762 + 1,161805*Hologic; FN BMD Lunar = 0,084782 + 1,116509*Hologic; forearm BMC Lunar = 0,148564 + 1,117715*Hologic; LS BMC Lunar = 7,143123 + 0,923483*Hologic; FN BMC Lunar = 0,079107+ 1,106219*Hologic; BA forearm Lunar = 0,784022 + 0,683982*Hologic; BA LS Lunar = 6,843735 + 0,765959*Hologic; BA FN Lunar = -0,467152 + 1,023246*Hologic. Following the BMD adjustments, *Z*-scores at each anatomical site were also calculated considering standard data reference ranges for gender and age provided by the Lunar manufacture (EE.UU. Lunar data reference for adults and BMDCS data reference for children adjusted for

height). This approach to calibrate different DXA models has been previously used and it is deemed acceptable for studies of this kind [101,102].

Statistical analyses

Each study of this thesis has its own statistical analysis; briefly:

Cross-sectional analysis Independent t-tests were used to compare descriptive characteristics and crude values of bone measurements between dance students and controls. Bone parameters were compared after adjustment for some covariates using a three-factor analysis of covariance (ANCOVA). All residuals were tested for normal distribution using the Kolmogorov-Smirnov test. Multiple regression analysis was performed to test for the association between bone measurements (dependent variable) with several independent variables known as predictors from literature.

Chi-square analyses were also performed to examine prevalence differences of low BMD between groups (dancers vs. controls). Logistic regression analyses were executed to investigate associations between low BMD and some covariates. For all analyses the SPSS - version 20.0 (IBM SPSS, Chicago, IL) was use while statistical significance was set at $p<0.05$.

Longitudinal analysis: Independent t-tests were used to compare general characteristics between vocational dance students and controls at each measured occasion (stratified by sex). Based on a multilevel approach applied to longitudinal data, SuperMix software (SSI - Scientific Software International, Inc.) was used to investigate the predictors of bone mass accrual over time in each anatomical site. Chronological age was used as the metric of time: time 0 corresponds to mean chronological age (on average around 12 years of age); negative values at X axis represents the number of years before mean chronological age, whereas positive values represent number of years after mean chronological age. The level of significance was set at $p<0.05$.

Genetic association study: Independent t-tests were used to compare general characteristics between dancers and controls (stratified by bone mass phenotypes) using the software SPSS (version 18.0). Hardy-Weinberg equilibrium (HWE) of alleles at individual loci (level of significance set at $p<0.01$) was measured at the level of the control population. Association of genotypes with study groups and independence of SNPs were assessed by logistic regression with the “SNPassoc” package implemented in R. Four hereditary models were considered in the analysis

(codominant, dominant, recessive and log-additive). The adjustment for multiple testing was performed by the false discovery rate (FDR) method. Haplotype frequencies were inferred using the “haplo.stats” package implemented in R. Haplotype association with the study groups (OR, 95% CI and *p* values) was assessed for those with a minimum haplotype frequency of 0.01 and using as reference the most frequent haplotype.

CHAPTER 5: BONE MASS IN FEMALE DANCE STUDENTS PRIOR TO PROFESSIONAL DANCE TRAINING: A CROSS-SECTIONAL STUDY (STUDY 1)

Parts of this chapter have been published in a peer-review journal entitled “Bone mass of female dance students prior to professional dance training: a cross-sectional study”; PlosOne, 2017;12(7):1-11. The author of this Thesis appears as the leading author.

ABSTRACT

Background Professional dancers are at risk of developing low bone mineral density (BMD). However, whether low BMD phenotypes already exist in pre-vocational dance students is relatively unknown.

Aim To cross-sectionally assess bone mass parameters in female dance students selected for professional dance training (first year vocational dance students) in relation to aged- and sex-matched controls.

Methods Thirty-four female selected for professional dance training (10.9yrs ±0.7) and 30 controls (11.1yrs ±0.5) were examined. Anthropometry, pubertal development (Tanner) and dietary data (3-day food diary) were recorded. BMD and bone mineral content (BMC) at forearm, femur neck (FN) and lumbar spine (LS) were assessed using Dual-Energy X-Ray Absorptiometry. Volumetric densities were estimated by calculating bone mineral apparent density (BMAD).

Results Dancers were mainly at Tanner pubertal stage I (vs. stage IV in controls, $p<0.001$), and demonstrated significantly lower body weight ($p<0.001$) and height ($p<0.01$) than controls. Calorie intake was not different between groups, but calcium intake was significantly greater in dancers ($p<0.05$). Dancers revealed a significantly lower BMC and BMD values at all anatomical sites ($p<0.001$), and significantly lower BMAD values at the LS and FN ($p<0.001$). When adjusted for covariates (body weight, height, pubertal development and calcium intake), dance students continued to display a significantly lower BMD and BMAD at the FN ($p<0.05$; $p<0.001$) at the forearm ($p<0.01$).

Conclusion Prior to undergoing professional dance training, first year vocational dance students demonstrated inferior bone mass compared to controls. Longitudinal models are required to assess how bone health-status changes with time throughout professional training.

KEYWORDS: BMD; bone mass; paediatric; vocational dance training; elite dance

INTRODUCTION

Osteopenia and osteoporosis are of major public health concern [103]. These conditions are characterised by low BMD and low bone mineral content (BMC), which lead to a fragile skeleton and increased risk of osteoporotic fractures [103]. Physical exercise is a key factor against the development of these conditions [9,10], particularly weight-bearing exercises during the developmental years [36].

Although it is generally accepted that a moderate active lifestyle improves bone health, the effects of elite physical performance on bone health are not entirely clear [40,104,105]. Elite sports may have inherent several specific characteristics that might induce either beneficial or deleterious impact on bone metabolism [106]. In aesthetic sports, where leanness and control of body weight are essential requirements, the training loads that typically promote bone formation may be annulled [83,86,107]. For instance, concerns have been voiced over the possible negative effects of elite dance training demands on bone metabolism [19,44,46]. Indeed, female elite professional dancers are exposed to high levels of artistic and physical fitness strains, whilst aesthetic build and low body weight are embraced in dance culture [13,108]. In accordance, the majority of relevant studies report that professional female dancers have low BMD compared to controls or normative values [109], which increases the risk of developing osteoporosis in later life.

Low BMD and osteoporosis in adulthood may have paediatric antecedents, as bone mass during growth is the foundation for the adult skeleton [110]. Nevertheless, whether BMD differences between dancers and non-dancers do exist prior to professional dance training has to be confirmed. The aim of the present study was to assess levels of areal and volumetric measures of bone mass in first year female vocational dance students (selected for professional training).

METHODS

Participants and study design

First year female students accepted in a vocational dance school (school that offers full-time dance training to become professional dancers; students have to audition for a place) during the academic years 2012/2013 and 2013/2014 were invited to participate in the study with no preliminary exclusion criteria; a total of 34 (70.8%)

volunteered. Control participants were recruited from a local state school by excluding those who participated or had previously participated in organised extracurricular physical activities; the total of 111 (28%) volunteered out of 391 eligible participants. Of these 111 students, 30 were female and had the same chronological age as our dance students, and, therefore, were enrolled in the study. Amenorrheic/ oligomenorrheic pupils were not excluded. The mean age of dance students and controls was 10.9±0.7 yrs and 11.1±0.5 yrs, respectively. Participants and their guardians signed informed consents after reading a written explanation of the study and discussion with the investigators. The study was approved by the ethics committee of the Regional Administration of Health of Lisbon (Proc.063/CES/INV/2012).

Female dance students started their professional training in September (25 students in 2012 and 9 in 2013) and were assessed in December 2012 and 2013, respectively. According to published reports, a period of 3 months of physical exercise is not sufficient to induce bone mass gains in paediatric populations [111–113].

All participants were involved in 2 hours of physical education exercise twice a week; dance students had also taken recreational dance lessons on a weekly basis (1.8±0.7 hours per week). All participants described themselves as white Caucasian. Within the population of 34 dance students available for assessment, all underwent anthropometric measures, participated in bone measurements and reported Tanner stage, age at menarche, and menstrual history, while 32 (66.7%) completed a dietary questionnaire. Similarly, all 30 controls underwent anthropometric measures, participated in bone measurements and reported Tanner stage, age at menarche, and menstrual history, while 29 (96.7%) completed a dietary questionnaire.

Anthropometry, maturation assessment, menstrual, energy expenditure, and nutritional analysis

Chronological age was obtained as decimal age (date of birth minus measurement date). Height and body mass were measured in t-shirt, shorts and bare feet using a stadiometer (Seca, Seca217 portable stadiometer, Hamburg, Germany) with accuracy of 0.1 cm and an electric scale (TANITA BC-418 MA Segmental Body Composition Analyser; Tanita Corporation, Tokyo, Japan) with an accuracy of 0.1 kg. All measurements were administered by the same investigator twice (mean of the

two measurements were recorded); if the difference between the two measurements was greater than 0.3, a third measure was obtained. Pubertal development was assessed using Tanner staging (breast and public hair stage in girls; genitalia and pubic hair stage in boys) by self-reporting. Standard line drawings and written descriptions were provided, and participants selected the picture that most accurately reflected their appearance [88]. All participants were presented with a questionnaire to determine age at menarche and regularity of menstrual cycles. Amenorrhea was defined as the absence of menses for three consecutive months, whereas oligomenorrhea was considered when menstrual cycles occurred at intervals of greater than 35 days. Nutrient intakes were recorded via a validated 3-day food diary [90]. Participants were asked to record all food and beverages consumed during two school days and one weekend day following appropriate instructions. The Food Processor SQL Edition, version 9.8.1 was used to estimate average energy and calcium intakes.

Bone status assessments

BMD (g/cm^2) and (BMC) (g) were determined for non-dominant forearm (33% radius), lumbar spine (L1-L4) (LS) and femoral neck (FN). To estimate volumetric density, bone mineral apparent density (BMAD) (g/cm^3) was calculated for all sites using previously described formulas [96]. Vocational dance students and controls were assessed in two different centres using Dual-energy X-ray absorptiometry (DXA). DXA scans of dance students were performed using a GE Lunar Prodigy whereas DXA scans of controls were performed using a Hologic (Discovery Wi). The same certified technician conducted all scans and analyses at both centres. Although previous studies have demonstrated a high correlation between Lunar and Hologic DXA BMD measurements [97–99], there is a tendency for Lunar model to inflate BMD values by 15% compared to Hologic [100]. Therefore, in addition to the daily calibration required from each DXA manufacturer, cross-calibration of the two scanners was conducted using a group of 20 independent participants. These participants were measured with both Lunar and Hologic within a period of 5 days. Regression equations using BMD from Lunar as dependent variable and BMD from Hologic as independent variable were performed from the data obtained from the participants included from cross-calibration. The correlation between the two DXA models were high (forearm BMD: r=0.96, adjusted r^2=0.93, std.

error of estimate=0.03; LS BMD: r=0.96, adjusted r^2=0.92, std. error of estimate=0.05; FN BMD: r=0.97, adjusted r^2=0.93, std. error of estimate=0.05; forearm BMC: r=0.98, adjusted r^2=0.96, std. error of estimate=0.09; LS BMC: r=0.96, adjusted r^2=0.92, std. error of estimate=4.41; FN BMC: r=0.94, adjusted r^2=0.88, std. error of estimate=0.46; bone area (BA) forearm: r=0.82, adjusted r^2=0.66, std. error of estimate=0.16; LS BA: r=0.88, adjusted r^2=0.76, std. error of estimate=4.64; BA FN: r=0.87, adjusted r^2=0.75, std. error of estimate=0.35). The Hologic BMD, BMC and BA data were further converted to the Lunar data using the following equations: forearm BMD Lunar = -0,085263 + 1,356535*Hologic; LS BMD Lunar = 0,030762 + 1,161805*Hologic; FN BMD Lunar = 0,084782 + 1,116509*Hologic; forearm BMC Lunar = 0,148564 + 1,117715*Hologic; LS BMC Lunar = 7,143123 + 0,923483*Hologic; FN BMC Lunar = 0,079107+ 1,106219*Hologic; BA forearm Lunar = 0,784022 + 0,683982*Hologic; BA LS Lunar = 6,843735 + 0,765959*Hologic; BA FN Lunar = -0,467152 + 1,023246*Hologic.

Statistical analyses

Projected power to detect differences between dance students and controls were performed based on prior studies with similar cohorts and study design. Power calculations were conducted based on a sample of female dance students (n=33, 16.2+2.0yr) and controls (n=90, 16.6+1.0yr) with BMD at the femoral neck as the main outcome. Assuming a detectable difference of 0.4 standard deviation and 90% power, calculations indicated that a sample of 50 volunteers was required for the present cross-sectional study (25 dance students and 25 controls).

Independent t-tests were used to compare descriptive characteristics and crude values of bone measurements between dance students and controls. Bone parameters were compared after adjustment for Tanner stage, height, body mass and calcium intake using a three-factor analysis of covariance (ANCOVA). All residuals were tested for normal distribution using the Kolmogorov-Smirnov test. As the normality assumptions were not violated in the majority of sites, multiple regression analysis was performed to test for the association between bone measurements (dependent variable) with several independent variables known as predictors from literature (maturation, body weight, height, energy and nutrition intakes); each variable was additionally inserted in the same model as a stepwise manner – one model for each anatomical site. For all analyses the SPSS - version

20.0 (IBM SPSS, Chicago, IL) was use while statistical significance was set at $p<0.05$.

RESULTS

Dance students revealed significantly lower body weight and height than controls ($p<0.001$ and $p=0.001$, respectively; Table 3). By the time of the assessment, two dance students and 15 controls reached menarche (one dancer had oligomenorrhea, whereas the other dancer and all 15 controls had regular menses). More dance students were at Tanner sexual pubertal development I (67.6%), while controls were at stage IV (40.0%); $p<0.001$). There was no significant difference in calorie intake between groups, but daily calcium intake was significant greater in dancers ($p=0.03$).

Table 3. Participant characteristics

	Dance Students (N=34)	Control Students (N=30)
Age (years) (1)	10.9 ± 0.7	11.1 ± 0.5
Height (cm) (1)	143.8 ± 6.8**	150.4 ± 9.7
Weight (Kg) (1)	33.0 ± 5.8***	48.2 ± 9.9
Dance training before vocational dance school (h/week) (1)	1.8±0.7	---
Age at menarche (2)	10.5 ± 0.7	10.8 ± 0.9
Amenorrhea (2)	0.0	0.0
Oligomenorrhea (2)	0.34	0.0
Calcium intake (mg/day) (1)	839.2 ± 498.1*	664.8 ± 331.7
Energy intake (Kcal/day) (1)	1863.8 ± 498.7	1763.0 ± 339.0
Tanner stage 1 (2)	67.6	6.7
Tanner stage 2 (2)	32.4	30.0
Tanner stage 3 (2)	0.0	23.3
Tanner stage 4 (2)	0.0	40.0

(1) Values are means + SD
(2) Values are percentages
* p<0.05; ** p<0.01; *** p<0.001

Dance students displayed significantly lower ($p<0.001$) crude BMD and BMC values than controls at all measured sites (Table 4). Significantly lower BMAD values by 43.6% were also found at the FN in dancers compared to controls ($p<0.001$; Table 2), and 17.6% lower at the LS ($p<0.001$; Table 4).

Table 4. Unadjusted bone parameters

	Dance Students (N=34)	Control Students (N=30)	Relative Difference (%)
Forearm measures			
BMC (g)	1.22 ± 0.22	1.69 ± 0.29***	38.1
BMD (g/cm^2)	0.54 ± 0.07	0.69 ± 0.07***	28.2
BMAD (g/cm^3)	0.24 ± 0.32	0.29 ± 0.34	18.6
FN measures			
BMC (g)	2.95 ± 0.69	3.67 ± 0.71***	24.5
BMD (g/cm^2)	0.81 ± 0.14	1.02 ± 0.09***	25.0
BMAD (g/cm^3)	0.19 ± 0.04	0.27 ± 0.04***	43.6
LS measures			
BMC (g)	29.07 ± 8.87	40.23 ± 11.38***	38.4
BMD (g/cm^2)	0.76 ± 0.14	0.98 ± 0.14***	28.3
BMAD (g/cm^3)	0.13 ± 0.04	0.15 ± 0.02***	17.6

Values are means + SD
* p<0.05; ** p<0.01; *** p<0.001
BMC= bone mineral content; BMD= bone mineral density; BMAD= bone mineral apparent density

Regression analyses revealed that when bone parameters were adjusted for body weight, height, pubertal development (Tanner test) and calcium intake, dance students continued to display significantly lower BMD at the forearm (p=0.02) and FN (p=0.04) (Table 5). For the same adjustments, BMAD values at the FN were also significantly lower in dancers than controls (p<0.001) (Table 5). The other remaining bone mass parameters were not significant different between dancers and controls after the adjustment (Table 5).

Table 5. Adjusted bone parameters for Tanner stage, height, body weight and calcium intake

	Dance Students (N=34)	IC 95%	Control Students (N=30)	IC 95%	Relative Difference (%)
Forearm measures					
BMC (g)	1.37 ± 0.06	1.25 – 1.49	1.56 ± 0.07	1.43 – 1.69	13.9
BMD (g/cm^2)	0.59 ± 0.02	0.56 – 0.62	0.66 ± 0.02**	0.62 – 0.69	11.9**
BMAD (g/cm^3)	0.26 ± 0.39	0.13 – 0.39	0.27 ± 0.07	0.13 – 0.41	3.8
FN measures					
BMC (g)	3.38 ± 0.12	3.12 – 3.64	3.28 ± 0.14	2.98 – 3.57	3.0
BMD (g/cm^2)	0.87 ± 0.03	0.82 – 0.92	0.96 ± 0.03*	0.92 – 1.04	10.3*
BMAD (g/cm^3)	0.19 ± 0.07	0.17 – 0.21	0.27 ± 0.01***	0.25 – 0.29	42.1***
LS measures					
BMC (g)	36.14 ± 2.04	32.05 – 40.22	32.47 ± 2.33	27.79 – 37.14	12.8
BMD (g/cm^2)	0.87 ± 0.03	0.82 – 0.93	0.86 ± 0.03	0.80 – 0.93	6.1
BMAD (g/cm^3)	0.14 ± 0.01	0.12 – 0.16	0.15 ± 0.01	0.13 – 0.17	7.1

Values are means + SD
* p<0.05; ** p<0.01; *** p<0.001
BMC= bone mineral content; BMD= bone mineral density; BMAD= bone mineral apparent density

Multiple regression analysis showed body weight and height to be significantly associated with BMC at the FN (R^2=0.607; p=0.03 and p=0.01, respectively), whist Tanner stage was significantly associated with BMD at the LS (R^2=0.654; p=0.002). Height was also a significant predictor of BMAD at the FN (R^2=0.637; p=0.02), and Tanner stage a significant predictor of BMC at the LS (R^2=0.600; p=0.02) and BMD

at the forearm (R^2=0.710; *p*=0.03). Calcium intake was significantly associated with BMAD measurements at the forearm (R^2=0.100; *p*=0.03).

DISCUSSION

To my knowledge, this is the first study which examined levels of areal and volumetric measures of bone mass in pre-pubertal female vocational dance students prior to undergoing any serious professional training. Present data revealed that first year vocational dancers demonstrated significantly lower adjusted BMD at the forearm, and significantly lower adjusted BMAD and BMD at the FN compared to aged- and sex-matched controls. It could be argued, therefore, that by the time our volunteers were selected to receive professional dance training they already demonstrated inferior bone mass measurements than controls.

It is difficult to compare the present findings with available data as the majority of the latter have examined professional dancers, non-elite adolescents or advanced vocational students [15,60,65,67]. This means that, unlike our participants, those involved in the aforementioned studies have already been exposed to the effects of dance training on the skeleton (they have been exercising for longer than two years). Also, previous studies on vocational dance students have reported mean ages of 16.7±0.8yr [19], 17.0±0.2yr [28,44], 21.5±3.7yr [43] and 20.7±1.8yr [16], which are significantly higher than our cohort (10.9±0.7yr), while only one of these studies had estimated volumetric densities [19]. This estimation may be particular relevant as it reduces the effect of bone size on areal density [96]. It is, therefore, important to calculate volumetric densities when interpreting paediatric densitometry in order to avoid overestimating bone mass values in tall children and underestimate it in short children [96,114]. Nevertheless, it should be highlighted that the studies on vocational dance students suggest that vocational dance training environment can lead to low body weight values, menstrual disturbances, diet restriction and, consequently, low bone weight phenotypes [19,43,44]. Indeed, participants involved in aesthetic activities, like elite dancing, have been identified as potentially at-risk to develop the female athlete triad [11,24]. However, our results might indicate the existence of a dance audition selection bias, and not potential deleterious effects of professional dance training volumes on bone metabolism.

Multiple regression analysis revealed that maturation and body type characteristics are likely to associate with bone mass parameters. Actually, the

majority of our dance students were at Tanner sexual stage I, whereas controls were at stage IV. Dancers were also significantly shorter and lighter than controls and only two had reached menarche, against 15 controls of the same age. Moreover, dancers had significant greater calcium intakes and total energy intake (despite not significant) than controls. Therefore, the present results may suggest that children with a predisposition for low body weight and delayed maturation are selected for professional dance training. Indeed, genetic factors seem to account for the majority of the bone mass phenotypes [115]. It was showed that girls who experience later menarche also have low values of BMD during pre-puberty [116], suggesting that genetics is a determinant factor. However, this issue needs to be further investigated in dancers. Longitudinal research protocols should be used to establish whether such bone mass values would have any bearing to peak bone mass, which is an important factor for prevention of bone fracture and osteoporosis [3,117].

One of the strengths of the current study is the representativeness of its sample given the relatively large number of vocational dance students who volunteered. This issue answers one of the main criticisms regarding dancers´ bone health which is related to the relatively small studied cohorts [109]. Another strength of this study is the young age of our participants; first year vocational dance students have never been studied before in relation to bone health prior to professional dance training. A further strength might be the confounding variables used to analyse bone mass results, which have not been frequently considered in the past [109]. This is also the case with the effect of bone size on DXA measurements; therefore, bone mass data presented in terms of BMC, BMD and BMAD is another strength of the present study.

It is reasonable to assume that the present results may have been influenced by methodological limitations. Due to the study's observational nature, causality and changes through time cannot be established. Self-reported pubertal development and self-reported nutrition data are also acknowledged as shortcomings. Finally, the use of two different DXA scans to assess participants' bone mass and the need to adjust the data for potential bias is a limitation. Nevertheless, this approach has been previously used and it is deemed acceptable for studies of this kind [101,102].

CONCLUSIONS

Prior to commencing full professional dance training, first year female vocational dance students demonstrate low bone mass parameters compared to aged- and sex-controls. Therefore, the low BMD values reported in professional dancers might have their genesis during the growing years. Further longitudinal research is required to ascertain how bone mass parameters change with time throughout professional dance training.

CHAPTER 6: PREVALENCE OF LOW BONE MINERAL DENSITY IN VOCATIONAL AND PROFESSIONAL BALLET DANCERS (STUDY 2)

Parts of this chapter have been published in a peer-review journal entitled "Prevalence of low bone mineral density in vocational and professional ballet dancers"; Osteoporosis International, 2017;28(10):2903-12. The author of this Thesis appears as the leading author.

ABSTRACT

Background The prevalence of low BMD in ballet dancers remains ambiguous.

Aim To determine the prevalence of low BMD in vocational dance students and professional ballet dancers, and its association with body weight, FM, LM, menarche and maturation.

Methods The total of 152 vocational dance students (112 girls, 40 boys) and 96 sex-aged-matched controls (56 girls, 40 boys), and 58 professional ballet dancers (40 female, 18 male) and 66 sex- aged-matched controls (46 female, 20 male) were assessed at the LS, FN, forearm and total body by DXA. Maturation and menarche were assessed via questionnaires.

Results The prevalence of low BMD in female VBD at the forearm was 9.2% (0% in controls, $p<0.01$) and 16.4% at the LS (5.5 % in controls, $p=0.05$). In female professional dancers the prevalence of low BMD at the forearm was 37.5% (17.4% in controls, $p<0.001$); 17.5% were osteoporotic. Female vocational dancers (reference group) were more likely to display low BMD at the forearm and LS than controls (OR= 0.1; $p<0.05$ and OR=0.2; $p<0.05$, respectively). FM was significantly associated with low BMD at the forearm in female vocational and female professional dancers (OR=0.9; $p=0.05$; OR=1.8; $p<0.01$, respectively). BM and LM were significantly associated with low BMD at the forearm in female professional dancers (OR=0.7; $p<0.05$ and OR=0.5, $p<0.01$, respectively). No significant differences were found between males.

Conclusions Compared to controls, the prevalence of low BMD is significantly higher at both impact and non-impact sites in female vocational dancers. Female professional dancers only have higher prevalence at non-impact sites. Body weight and FM seem to be associated with these findings.

KEYWORDS: bone mass; prevalence; associated factors; elite dance; ballerinas

INTRODUCTION

Osteoporosis and osteopenia (i.e. BMD) are recognised as the most frequent bone disorders, linked to high treatment costs and limited quality of life [118,119]. Hence, the identification of those at high-risk is crucial for planning appropriate prevention programmes. The diagnosis of low BMD in premenopausal women and children is based on the ISCD guideline, whereas a diagnosis is confirmed when BMD values lie within 2.0 standard deviations (SD) or more below the average value [120]. The ACSM has proposed different guidelines for the diagnosis in athletes. The term “low BMD” is used for BMD values between -1.0 and -2.0 SD, and the term “osteoporotic” for BMD equal or less than -2.0 SD (along with secondary risk factors for stress fractures) [11].

Low BMD has been traditionally associated with elderly and postmenopausal women [121], though some athletic populations, as endurance athletes, might also be at increased risk [122,123]. In ballet dancers, however, aspects regarding low BMD remain ambiguous [109]. While some authors underline the negative effects of professional dance training on bone metabolism (e.g. lean body type required for performance) [18,19,25], others suggest that the mechanical impact from dancing may provide a protection against low BMD, particularly at impact sites [27,47,48]. For instance, the high levels of muscular strength required for technical performance and weight-bearing activity associated with jumping may stimulate bone-forming cells [27,47,48]. Nevertheless, most of the relevant publications on ballet dancers have been categorised average of low quality [109]. The aims of the present study were to determine a) the prevalence of low BMD in vocational and professional ballet dancers, and b) its association with body weight, FM, LM, menarche and maturation.

METHODS

Study population

This study was conducted by inviting active students from vocational dance schools (children undergoing 4-8 hours a day dance training in order to prepare for the profession) and active dancers from professional ballet companies. Pilot studies were administrated at a vocational dance school and a professional ballet company in order to calculate the sample size needed for prevalence estimate; sex and aged matched controls were also included in both cases. In a sample of 36 female vocational dance students and 36 matched-controls, low BMD (*Z*-score of <-2.0) LS

was found in 36% and 6%, respectively. Based on this finding, we estimated that 37 participants were needed in each group to obtain 90% power, with α=0.05. Similarly, in a sample of 22 female professional ballet dancers (22 matched-controls) and 10 male professional dancers (10 matched-controls), the prevalence of low BMD (*Z*-score of -1.0) at the LS was found to be 32% (vs. 5%) in female dancers and 20% (v. 0%) in male dancers. We subsequently estimated that 42 female participants and 46 male participants in each group were needed to reach significance (90% power, α=0.05). Assuming participants´ non-response and possible dropouts, we approached two vocational dance schools and two professional ballet companies.

From the total of 502 participants (362 vocational and 140 professional), 158 vocational dance students and 63 professional ballet dancers volunteered. From this cohort, those who had received or were receiving medications known to affect bone metabolism were excluded (one professional dancer), together with those receiving calcium supplements (two vocational and one professional dancer). Given the differences in bone mass values between individuals from different races [81], only participants referring themselves as white European-Caucasian dancers were included. Based on these criteria, the total of 152 vocational (112 girls, 40 boys) and 58 professional ballet dancers (40 female, 18 male) were finally included in this study. Participants provided details on physical exercise (hours per week). Female and male vocational dancers reported to perform 18.2±7.0 and 19.5±7.2 hours per week of dance training, respectively. Female and male professional dancers reported 24.9±4.4 and 28.1±3.1 hours per week of dance training, respectively. Details of the recruited dance population and its participation rate appear in Figure I.

Controls were also included in this study. Controls for the VBD were recruited from two local state schools, while controls for professional dancers were recruited from two local state universities. Eligibility criteria for controls were set according to dancers' characteristics, i.e. controls were only considered eligible if they were of the same sex, age (defined as decimal age; 12-months difference of a dancer) and race (white European-Caucasian). Exclusion criteria included current and previous participation in regular and organised physical activities. This rule did not apply to children participants involved in physical education sessions at their school. Control participation was also restricted to those who had received or were receiving medications known to affect bone metabolism. All participation criteria explaining the purpose for the recruitment was advertised via email and letters, following consent

from the respective boards of directors. Out of the 282 responses (105 pupils, 177 university students), 256 fulfilled the current criteria and were included in the study [controls for vocational dancers: 96 (56 girls, 40 boys); controls for professional dancers: 66, 46 female, 20 male]. Female and male controls for vocational dancers were involved in 2.4±0.5 and 2.1±0.4 hours per week of physical exercise, consisting mainly of school physical education. Female and male professional dancers controls did not report extra physical exercise apart from daily life routines.

All participants provided signed informed consent. They also underwent anthropometric measures, completed a menstrual questionnaire (non-validated questionnaire; participants were asked about their age at menarche and regularity of menstrual cycles) and participated in bone/body composition measurements (Figure 6). All procedures were approved by the NHS Health Research Authority, UK (Proc.14/WM/0008 and 14/WM/0009) and by the ethics committee of the Regional Administration of Health of Lisbon, Portugal (Proc.063/CES/INV/2012) in accordance with the Helsinki Declaration.

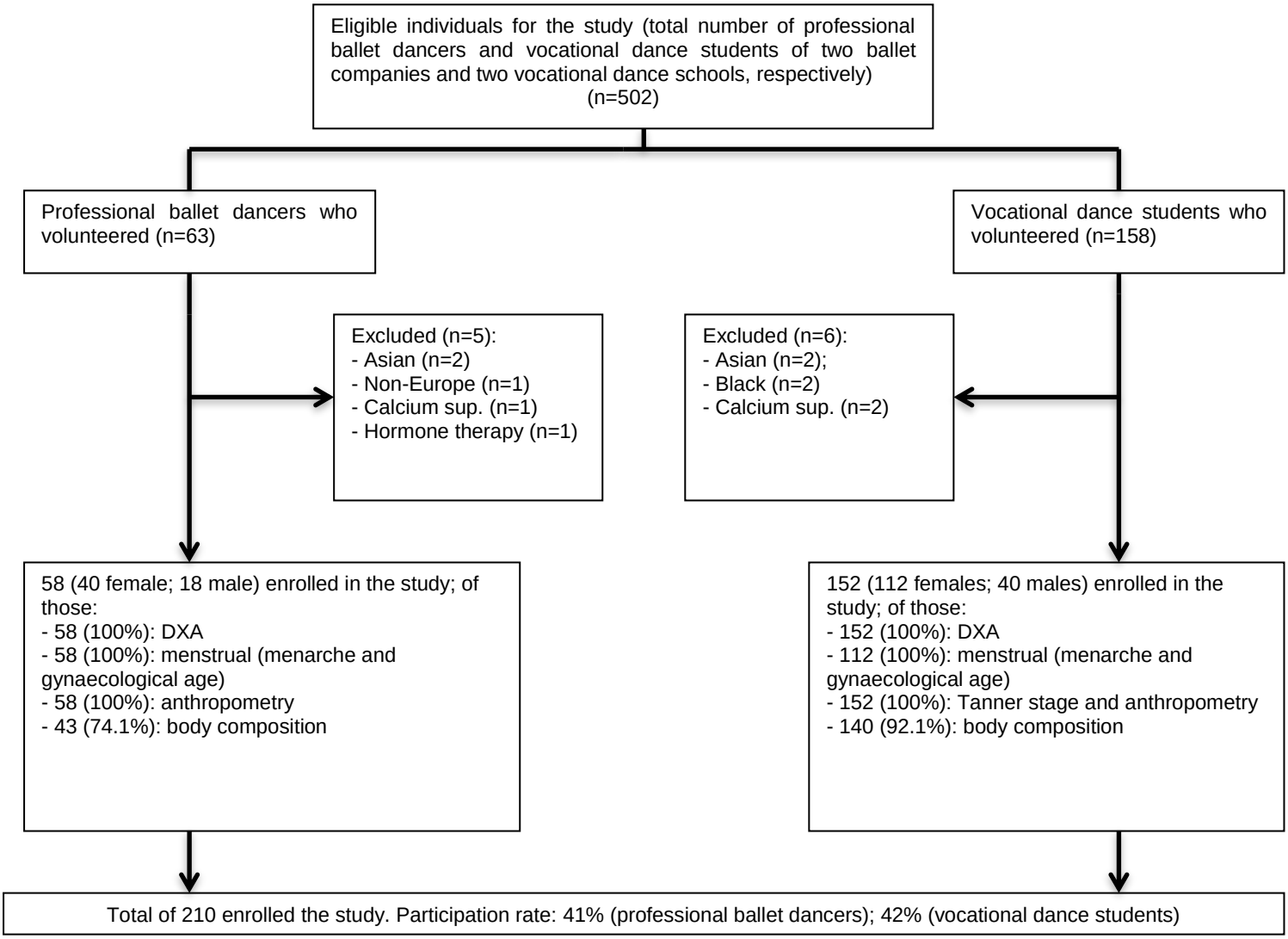


Figure 6. Enrolment of dance population in the study.

Anthropometry measurements, menstruation, smoking, nutrition intake, hormonal analysis and pubertal assessment

Chronological age was obtained as decimal age (date of birth minus measurement date). Participants' height (m), sitting height (m) and BM (kg) were measured using standard stadiometers (Seca) and digital scales (Tanita), respectively. BM index (BMI) was calculated as kilograms per square meter ($kg.m^{-2}$). Female participants completed a questionnaire to determine age at menarche. Total lifetime menses (number of menses since menarche to current age) were calculated as previously described [124]. Primary amenorrhea was defined as the absence of menarche by the age of 15 [125]. Gynaecological age (years) was calculated from the year of menarche to the age at which data were collected – current age [126]. Pubertal

development in vocational dance students and their controls were self-reported using the Tanner sexual staging questionnaire [88].

Definition of low BMD

The ISCD criterion for children was used to assess VBD and their controls; the ISCD has adopted the term “low BMD” for a *Z*-score less than -2.0 [120]. The ISCD guidelines were also considered for adult controls; the term “low BMD” for a Z-score equal or less than -2.0 was used [11]. The ACSM guidelines were adopted for PBD, since dancers are “performing athletes” [11,13]. The ACSM uses the term “low BMD” for a *Z*-score between -1.0 and -2.0 (along with secondary risk factors for stress fractures) and the term “osteoporotic” for a *Z*-score equal or less than -2.0 (along with secondary risk factors for stress fractures) [11]. Given their well-know association with low BMD [127,128], the secondary osteoporosis risk factors considered in this study were primary amenorrhea and/or low body weight (defined as a body weight index of <18.5). According to the ACSM, these risk factors might also be an indicator of hypogonadism, also known to be associated with increased risk for fractures [11].

Body composition and bone measurements

BMD at the LS, FN and forearm (1/3 distal radius) were measured using DXA. Body composition was assessed through a DXA whole-body scan [FM and LM (Kg)]. As participants were from different regions, two different DXA devices were used [Hologic (Discovery Wi) and Lunar (GE Lunar Prodigy)].

It is known that Lunar and Hologic BMD measurements demonstrate high correlation values between them [98,100]. It is also known that there is a tendency for Lunar model to inflate BMD values compared to Hologic [100]. Therefore, besides the daily calibration required from each DXA manufacturer, cross-calibration of the two scanners was also conducted on a group of 20 men and women; the age of these 20 participants covered the age-range of the entire sample (both dancers and controls) used for the purpose of the present study. The 20 participants were measured with both Lunar and Hologic within a period of 5 days. Subsequently, regression equations using BMD from Lunar as dependent variable and BMD from Hologic as independent variable were performed taking into account cross-calibration. The correlation between the two DXA models were high (forearm BMD: r=0.96, adjusted r^2=0.93, std. error of estimate=0.03; LS BMD: r=0.96, adjusted

r^2=0.92, std. error of estimate=0.05; FN BMD: r=0.97, adjusted r^2=0.93, std. error of estimate=0.05). The Hologic BMD data were further converted to the Lunar data using the following equations: Forearm BMD Lunar = -0,085263 + 1,356535*Hologic; LS BMD Lunar = 0,030762 + 1,161805*Hologic; FN BMD Lunar = 0,084782 + 1,116509*Hologic. After all these BMD adjustments, the *Z*-scores at each anatomical site were calculated considering standard data reference ranges for gender and age provided by the Lunar manufacture (EE.UU. Lunar data reference for adults and BMDCS data reference for children adjusted for height).

Statistical analyses

Independent t-tests were used to compare general characteristics between dance population and controls. Chi-square analyses were performed to examine prevalence differences of low BMD between groups (dancers vs. controls). These analyses were conducted separately in vocational dancers and professional dancers, stratified by sex in each group. Linear regression analysis and logistic regression analyses (also separating vocational and professional) were conducted to investigate associations between low BMD (dancers as the reference group) and the following variables: body weight, age at menarche, gynaecological age, FM, LM, Tanner stage and energy intake. Since our dancers were recruited from different ballet schools/companies and were scanned using two DXA devices, all analyses were conducted controlling for a potential school/company and DXA effects. Data are presented as odds ratio (OR) and 95% confidence intervals (95% CI). Statistical significance was set at $p<0.05$.

RESULTS

Table 6 depicts the general characteristics of all participants. Table 6 indicates that maturity differences between dancers and controls are more pronounced in female vocational dancers than their male counterparts. Compared to controls, female and male vocational dancers revealed significantly lower body weight (10.8Kg and 11.1Kg less, respectively; $p<0.001$), BMI (4.39Kg/m^2 and 3.59Kg/m^2 less respectively; $p<0.001$) and FM (9Kg and 8Kg less, respectively; $p<0.001$). In female vocational dancers, age of menarche was ~18 months later than controls ($p<0.001$). Similarly, female and male professional dancers revealed significantly lower body weight (9.4Kg and 7.0Kg less; $p<0.001$ and $p<0.01$, respectively) and BMI (3.9Kg/m^2 and 2.3Kg/m^2 less; $p<0.001$ and $p<0.05$, respectively) compared to controls. Female

professional dancers also demonstrated significantly lower FM (10.2Kg less, $p<0.001$) and had their menarche approximately two years later than controls ($p<0.001$). BMD values adjusted for some covariates (body weight, DXA-device, school/company, and pubertal development - in case of vocational dancers and matched controls) was significantly lower in female vocational dance students at the forearm ($p<0.001$), and at the FN and forearm in male vocational dance students ($p<0.01$ and $p<0.001$, respectively). Female professional dancers revealed significantly lower adjusted BMD at the forearm ($p<0.05$) compared to controls, whereas male professional dancers showed significantly adjusted BMD values than controls at the FN and LS ($p<0.01$ and $p<0.001$, respectively) (Table 6).

Table 6. General characteristics of the study population

	Vocational ballet dancers (N= 152)		Children controls (N= 96)		Professional ballet dancers (N= 58)		Adult controls (N= 66)	
	Girls (N= 112)	Boys (N=40)	Girls (N= 56)	Boys (N= 40)	Female (N=40)	Male (N=18)	Female (N=46)	Male (N= 20)
Age (yrs.)[(1)]	13.5 ± 2.4	13.2 ± 2.2	13.7 ± 2.2	13.3 ± 2.0	33.2 ± 11.5	28.4 ± 7.0	30.2 ± 10.2	27.5 ± 6.8
Height (cm)[(1)]	155.8 ±10.4	156.1 ± 15.5	155.3 ± 8.7	160.5 ± 10.3	163.4 ± 5.0	176.4 ± 3.9	162.1 ± 6.4	177.0 ± 5.9
Body weight (Kg)[(1)]	42.1 ± 8.7	44.3 ± 13.2	52.9 ± 12.3***	55.4 ± 13.1***	49.9 ± 4.9	70.4 ± 5.4	59.3 ±9.2***	77.4 ± 10.4**
BMI (Kg/m^2)[(1)]	17.4 ± 5.7	17.8 ± 2.1	21.7 ± 3.8**	21.3 ± 3.9***	18.6 ± 1.5	22.5 ± 1.7	22.5 ± 3.5***	24.8 ± 4.0*
Lean mass (Kg)[(1)]	30.9 ± 6.5	34.3 ± 11.7	31.6 ± 4.9	36.5 ± 9.6	36.7 ± 3.6	57.0 ± 4.9	35.6 ± 3.6	58.2 ± 9.1
Fat mass (Kg) [1)]	8.3 ± 17.3	6.7 ± 3.1	17.3 ± 6.1***	14.7 ± 7.2***	10.2 ± 3.7	11.0 ± 5.7	20.0 ± 7.8***	14.9 ± 7.6
Age menarche (yrs.)[(1)]	12.7 ± 2.2	-	11.1 ± 1.1***	-	13.6 ± 1.9	-	11.7 ± 1.2***	-
Gynaecological age (yrs.)	2.3 ± 1.8	-	3.4 ± 2.0**	-	19.2 ± 11.7	-	18.7 ± 10.5	-
Primary amenorrhea[(2, 3)]	3.9	-	0	-	27.5	-	2.4**	-
Tanner I[(4)]	37.0	30.0	1.9***	0.0***	-	-	-	-
Tanner II[(4)]	23.1	45.0	14.8***	22.4***	-	-	-	-
Tanner III[(4)]	28.7	7.5	18.5***	34.7***	-	-	-	-
Tanner IV[(4)]	9.3	12.5	40.7***	34.7***	-	-	-	-
Tanner V[(4)]	1.9	5	9.3***	6.7**	-	-	-	-
Physical exercise (h/week)	18.2 ± 7.0	19.5 ± 7.2	2.4 ± 0.5***	2.1 ± 0.4***	24.9 ± 4.4	28.1 ± 3.1	-	-
BMD FN (g/cm^2)	0.95 ± 0.01	0.97 ± 0.03	0.98 ± 0.02	1.08 ± 0.02**	1.07 ± 0.04	1.20 ± 0.03	1.02 ± 0.03	1.04 ± 0.03**
BMD LS (g/cm^2)	0.93 ± 0.01	0.91 ± 0.01	0.94 ± 0.02	0.92 ± 0.02	1.14 ± 0.03	1.23 ± 0.03	1.06 ± 0.03	1.06 ± 0.03***
BMD FA (g/cm^2)	0.63 ± 0.07	0.63 ± 0.01	0.70 ± 0.01***	0.69 ± 0.01***	0.69 ± 0.02	0.84 ± 0.02	0.75 ± 0.02*	0.81 ± 0.02

[(1)] Values are means ± SD
[(2)] Values are percentages; express the percentage of dancers/controls with amenorrhea or oligomenorrhea by the time of the assessment (current menstrual status)
[(3)] Value is percentage; express the percentage of dancers/controls with primary amenorrhea out of those that have reached the menarche by the time of the assessment
[(4)] Value is percentage; express the percentage of vocational ballet dancers/controls at each Tanner stage by the time of the assessment
BMD values were adjusted for body mass, DXA-device, school/company, and pubertal development (in case of VBD and matched controls)
* p<0.05, **p<0.01, ***p<0.001; dancers (both vocational and professional) significant different from controls
MD = bone mineral density; FN = femoral neck; LS = lumbar spine; FA = forearm

Tables 7 and 8 show the prevalence of low BMD. Significantly higher prevalence of low BMD at the forearm (9.2% vs. 0%, p=0.01; Table 7) and LS (16.4% vs. 5.5%, p<0.05; Table 7) was noted in female vocational dancers compared to controls. It was also found a significant higher prevalence of low BMD at the forearm (37.5% vs. 17.4%, p<0.001; Table 8) in our female professional dancers than their controls. Based on the ACSM criteria, 17.5% female professional dancers were osteoporotic at the forearm (Table 8).

Table 7. Prevalence of low bone mineral density in vocational dancers and aged- and sex-matched controls

	ISCD criteria					
	LS (%)		FN (%)		FA (%)	
	Female	Male	Female	Male	Female	Male
Vocational dancers	16.4	7.5	8.3	10.5	9.2	2.5
Controls	5.5*	2.1	12.7	4.2	0.0**	2.2

Values are percentages
*p<0.05, **p<0.01, ***p<0.001; dancers significant different from controls
LS = lumbar spine; FN = femoral neck; FA = forearm
International Society of Clinical Densitometry (ISCD) criteria was to assess vocational dancers and aged- and sex-matched controls: "low BMD" was defined for a *Z*-score less than -2.0

Table 8. Prevalence of low bone mineral density in professional dancers and aged- sex-matched controls

	LS (%)		FN (%)		FA (%)	
	Low BMD	Osteoporotic	Low BMD	Osteoporotic	Low BMD	Osteoporotic
Female dancers(1)	10.0	2.5	12.5	0.0	37.5	17.5
Female controls(2)	6.5	-	8.7	-	17.4***	-
Male dancers(1)	16.7	0.0	0.0	0.0	11.1	5.6
Male controls(2)	2.5	-	10.0	-	2.0	-

Values are percentages
* p<0.05, **p<0.01, ***p<0.001; dancers significant different from controls
LS = lumbar spine; FN = femoral neck; FA = forearm
(1)American College of Sports Medicine (ACSM) criteria was used to diagnose professional dancers: the term "low BMD" was applied for a *Z*-score between -1.0 and -2.0 alongside with osteoporosis risk factors (low body mass and/or presence of current menstrual disturbances); the term "osteoporosis" was applied to a *Z*-score less than -2.0 alongside with osteoporosis risk factors (low body mass and/or menstrual disturbances). In case of male dancers, the risk factor used was only low body mass.
(2)International Society of Clinical Densitometry (ISCD) criteria was used to diagnose controls: the term "low BMD" was applied for a Z-score less than -2.0.

Table 9 illustrates logistic regression analysis using *Z*-score as dependent variable (low BMD vs. normal BMD). Female vocational dancers were more likely to develop low BMD at the LS (OR=0.2; 95% IC, 0.08-0.78; p<0.05) and forearm (OR=

0.1; 95% IC, 0.11-0.67, p<0.05). Low BMD at the forearm in female vocational dancers were significantly associated with FM (OR=0.9; 95% IC, 0.77-1.00, p=0.05). Tanner stages were not significantly associated with low BMD at both LS and forearm. In female professional dancers body weight, FM and LM mass were significantly associated with increased odds of LBMD at this site (OR=1.8; 95% IC, 1.19-2.80, p<0.01; OR=0.7; 95% IC, 0.47-0.99, p<0.05; OR=0.5; 95% IC, 0.35-0.83, p<0.01, respectively). Neither age at menarche nor gynaecological age were associated with low BMD (p>0.05).

Table 9. Logistic regression analysis of associated factors for low BMD in vocational female dancers and professional female dancers

	Low BMD FA (VBD)			Low BMD LS (VBD)			Low BMD FA (BPD)		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Group (dancers vs. controls)	0.1	0.16 – 0.98	0.05	0.2	0.08 – 0.75	0.01	0.9	0.18 – 4.15	0.86
Body mass	1.3	0.98 – 1.82	0.06	1.0	0.97 – 1.05	0.72	1.8	1.19 – 2.80	0.01
Fat mass	0.5	0.25 – 0.87	0.02	1.1	0.83 – 1.38	0.60	0.7	0.47 – 0.99	0.05
Lean mass	1.0	0.91 – 1.11	0.93	0.9	0.92 – 1.08	0.89	0.5	0.35 – 0.83	0.01
Tanner stage	1.3	0.73 – 2.18	0.40	1.3	0.98 – 1.66	0.07	–	–	–
Age at menarche	1.1	0.78 – 1.45	0.68	0.9	0.51 – 1.41	0.53	0.9	0.70 – 1.41	0.97
Gynaecological age	0.8	0.51 – 1.15	0.99	0.7	0.64 – 1.40	0.77	0.9	0.94 – 1.06	0.95

Odds ratio (OR) and 95% CI estimated with a Z-Score of -2.0 or lower (for VBD) and a Z-Score of -1.0 or lower (for professional ballet dancers) as dependent variable. Reference group: vocational and professional dancers
BMD = bone mineral density; FA = forearm; LS = lumbar spine; VBD = vocational dance students; PBD = professional ballet dancers
Logistic regression analysis at femoral neck (both vocational and professional) and lumbar spine (professional) did not revealed significant differences between dancers and controls.

DISCUSSION

Data on low BMD prevalence in dancers has been ambiguous thus far. This is supported by a recent systematic review highlighting the need for further research [109]. To my knowledge, the present study is the first to provide prevalence estimates of low BMD in a relatively large cohort of ballet dancers. Previous reports on the field used BMD as main outcome [18,19,25,27,47,48], which do not allow discrimination of the true low BMD prevalence nor to address whether dancers represent a special population at increased risk for low BMD. The present study main finding was the significantly higher low BMD prevalence at the forearm (in female vocational students and professional dancers) and at LS (in female vocational

dancers) compared to matched-controls. It is noteworthy that the current female vocational dance students demonstrated higher odds for low BMD at both impact (LS) and non-impact sites (forearm). It was not found any difference regarding male professional dancers. The latter confirms previous data [129] and could be partly explained by the fact that males have less pronounced endocortical resorption and higher periosteal expansion compared to females [130].

Dancing has been considered as a weight-bearing activity [48]. Studies using weight-bearing physical activities have shown positive effects on bone mineral accrual in both adults and children [10,39]. Indeed, it has been suggested that 60 min x 3 a week of weight-bearing exercise is sufficient to prevent low BMD in general population [131]. Since the present participants were vocational and professional dancers, they were involved in daily classes of several hours of weight-bearing activity [14,82]. Considering data on bone cell biology and function of osteocytes as mechanosensory cells [87,132], it would be expected a significantly lower prevalence of low BMD in both vocational and professional dancers at impact sites and similar prevalence values at non-impact sites compared to controls. However, dancing is also an aesthetic activity whereas body size is essential for performance. This requirement might place dancers at risk for low body weight, a well-known risk factor for low bone mass phenotypes. Indeed, in the present study, both vocational and professional female dancers had significantly lower body weight values compared to their controls. However, the fact that female professional dancers revealed similar prevalence estimates at impact sites compared to controls might indicate that dance training is able to stimulate bone gains, even in the presence of lower body weight. Nevertheless, it seems that the same effects could not be seen in female vocational dancers since, these participants revealed higher prevalence estimates at both impact (LS) and non-impact sites. As LS is mainly constituted by trabecular bone (known to be more sensitive to mechanical stress from exercise [133]), and as ballet dancing requires high levels of muscular strength (placing considerable mechanical stress on lower back [13,82]), it is surprising to find a significantly higher number of cases with low BMD at this anatomical site in female vocational dancers. The reasons for these findings are currently unclear. Factors such as low energy availability, genetics and/or hormonal levels should be considered in future studies, given their association with low bone mass phenotypes [11,134]. Furthermore, since

our participants were exercising for many hours per week, the hypothesis that bone cells might be saturated due to dance training loading should not be excluded.

Another interesting finding was the higher prevalence of low BMD at impact sites (FN and LS) in male vocational dance students compared to controls (although not significant). Previous studies usually focus in female dancers as it is generally accepted that females have increased odds for low BMD. However, the present study suggests that young male dance students may also be at risk for low BMD. Indeed, adjusted BMD values for body weight and maturation were also found to be significantly lower than controls at both impact and non-impact sites. Future studies should also considerer young male dancers in relation to BMD in different settings.

Not all traditional osteoporosis risk factors were associated with the prevalence of low BMD in our sample. Body weight was significantly associated with low BMD in professional dancers at the forearm, and FM significantly associated with low BMD in vocational dancers at the same anatomical site. These findings were partly expected as BM and FM are predictors of low BMD [110,127]. LM is also known to be a predictor of low BMD in children [135]; nevertheless, we did not find any association between these two parameters. Actually, the present study was unable to identify factors associated with low BMD at the LS in female vocational dancers. Previous studies revealed that athletes with oligo/amenorrhea (both primary and secondary) have lower *Z*-scores compared to their eumenorrheic counterparts [25,126]. Although it was not investigated the association between menstrual history and bone phenotypes, no associations between low BMD and age at menarche or gynaecological age have been observed. Delayed puberty has also been linked with low BMD in children and adolescents [22,136]. No association between low BMD with Tanner staging was found, despite the fact that a significant difference between vocational students and controls was detected for sexual maturation.

The current results regarding low BMD in vocational dance students might be of concern, as young dancers may enter adulthood with relatively low BMD, which may further impair the peak bone mass attainment [3]. However, findings in children should be interpreted with caution due to biological changes which occur during growth [3]. Longitudinal studies should be conducted in vocational dancers to ascertain how bone mass changes throughout growing.

The clinical significance of low BMD lies on the increased risk of fracture [11,120]. It was not recorded fractures or injuries among our studied population.

Nevertheless, recent data have shown that over one year period the incidence of injury in vocational dance students was 1.42 per student and the risk of injury 76% [137]. Also, in professional baller dancers, a total of 355 injuries were recorded during a year, with an overall incidence of 6.8 injuries per dancer [84]. However, to our knowledge, there are no available data on the association between dance injuries and low BMD [109]. Notwithstanding, the prevalence of *Z*-scores below -1.0 is significantly higher among our dance population compared with controls. Indeed, since athletes in weight-bearing sports usually have 5-15% higher BMD than non-athletes [11], the ACSM emphasizes that a BMD *Z*-score of < -1.0 in athletic populations should be further investigated, even in the absence of fractures [11]. However, to the best of our knowledge, there are no preventative/screening measures in dance population regarding overall dancers' bone health yet.

It is reasonable to assume that the present study might have been influenced by methodological limitations such as the use of a self-reported questionnaire to assess age at menarche, gynaecological age and Tanner stage. I also acknowledge the lack of injury and fracture records for our participants as well as alcohol intake. Another limitation may be that the current data incorporate dancers born and raised in north or south Europe; I further recognise the potential selection bias of the current participants since they were recruited from different geographic regions.

CONCLUSIONS

Compared to controls, the prevalence of low BMD was significantly higher at lumbar spine (impact site) and forearm (non-impact site) in vocational female ballet dancers. This was the case only at non-impact site in professional female dancers. Body weight and fat mass seem to be associated with these findings. Future studies should explore the underpinning mechanisms associated with low bone mass phenotypes in dancers.

CHAPTER 7: ASSOCIATIONS BETWEEN OESTROGENS, GROWTH HORMONE, AND INSULIN-LIKE GROWTH FACTOR I WITH BONE MASS ACQUISITION IN FEMALE VOCATIONAL DANCE STUDENTS: A MIXED-LONGITUDINAL STUDY (STUDY 3)

Parts of this chapter have been submitted in the peer-reviewed journal *Journal of Bone and Mineral Research*. The author of this Thesis appears as the leading author.

ABSTRACT

Background There is a dearth of relevant research regarding the factors associated with low bone mass phenotypes in vocational dance students and how bone mass develops as they progress on their growth and professional training.

Aim To investigate BMC and BMD accruals in female vocational dance students and their association with circulating levels of oestrogens, GH and IGF-1.

Methods Sixty-seven vocational female dancers and 68 aged- and sex- matched controls (12.1±1.9yrs and 12.7±2.0yrs at baseline, respectively) were followed for three years. BMC and BMD were measured annually at impact sites (FN and LS), and non-impact site (forearm) using DXA. Anthropometry, age at menarche (questionnaire) and hormone serum concentrations (immunoradiometric assays) were also measured for the same period.

Results During the follow-up, female vocational dancers always had consistently reduced BMD values at all anatomical sites (p<0.001) and body weight (p<0.001) compared to controls. Serum IGF-1 concentrations were significantly increased in dancers compared to controls at 2yr follow-up (p<0.001). Menarche, weight, GH and IGF-1 were significantly associated with bone mass changes over time (p<0.05). However, these factors did not explain group differences in terms of BMC gains at impact sites (p>0.05). At the forearm, body weight was able to explain differences between groups in terms of BMC gains.

Conclusion As young female dancers progress on their professional training, their bone mass remains lower compared to controls at both impact and non-impact sites. Endocrine mechanisms do not seem to explain group differences in terms of bone mass gains.

KEYWORDS BMC; BMD; follow-up; ballet; athletic population

INTRODUCTION

Bone tissue is constantly renewed by the coordinated activity of osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells) in a process known as bone remodelling [138]. This process is regulated by local factors as well as systemic hormones [139] [140]. For example, the release of growth hormone-releasing hormone (GHRH) in the hypothalamus stimulates the production of GH from the pituitary gland [139]. Growth hormone acts on its primary target - the liver - where it stimulates the production of IGF-1 [140]. The interdependent roles of the aforementioned hormones in the regulation of bone remodelling have been well established [140]; GH and IGF-1 regulate bone cells by enhancing osteoblast activity [141], reducing osteoblast apoptosis and promoting osteoblastogenesis through stimulation of Wnt/β-catenin activity [140]. In contrast, the lack of GH secretion and low circulating levels of IGF-1 are usually associated with low BMD [22,142].

GH and IGF-1 activities decrease with age [143], but they are particularly up-regulated during the growing years [22,144]. Rising levels of gonadal steroids (specifically oestrogens) during growing (particularly during early puberty) are followed by rising activity of GH and IGF-1 [144]. This means that endocrine mechanisms during adolescence and puberty constitute important determinants of bone mass acquisition [2]. In accordance, delayed puberty has been reported to be associated with low BMC in children and adolescents [22]. Considering young dancers involved in rigorous training regiments, studies have shown that these participants have delayed puberty [145]. Additionally, low bone mass values at both impact and non-impact sites have also been reported in young dancers [19]; yet, less is known on the factors associated with low bone mass phenotypes on these populations (particularly at impact sites) [18,25,43,146]. Therefore, the aims of the present study were to: 1) model BMC and BMD accruals in female vocational dance students and matched-controls, and 2) determine whether circulating levels of oestrogens, GH and IGF-1 explain differences in bone mass gains between female vocational dance students and non-exercising adolescents.

METHODS

Participants' recruitment

Projected power to detect differences between dance students and controls was based on a prior study that has measured cross-sectionally first year female

vocational dance students (n=34, 10.9±0.7yr) and matched controls (n=30, 11.1±0.5yr); BMC at the FN (dancers: 2.95±0.69g/cm^2; controls: 3.67±0.7169g/cm^2) was selected as the main outcome given that literature suggests that in paediatric populations analysis on BMC outcomes should take precedence over BMD [147,148]. Assuming a 5% error and 90% power, calculations indicated that a sample of 40 volunteers was required for the present longitudinal study (20 dance students and 20 controls).

To recruit female vocational dance students, an introductory letter explaining the purposes of the study was sent to the executive boards of a vocational dance school that offers full-time dance training to become professional dancers with 4-8 hours a day of dance training. Following boards' agreement, the research team exposed the purposes of the study to vocational dancers and their guardians. From a total of 106 female students that were enrolled at the vocational school in the academic year 2012/2013, 67 (63.2%) volunteered. All these completed a questionnaire concerning their ethnicity, physical activity, medical history, and past/current calcium/vitamin D supplementation. As none of the volunteers reported consumption of medications/supplementation known to influence bone metabolism, nor reported illnesses/treatments that might affect bone metabolism, all 67 were enrolled in the study [all described themselves as white European-Caucasian, and were involved in 16.3±6.5 hours of dance training per week during 5 to 6 week days].

Female children and adolescent non-dancers were also recruited from two random local state schools to act as controls. Eligibility criteria for controls were set according to dancers' characteristics (i.e. age and race). Exclusion criteria included those who participated or had previously participated in sport activities outside their school curriculum. Control participation was also restricted to those who had received/were receiving medications known to affect bone metabolism and to who reported illnesses/treatments that might affect bone metabolism. Following consent from the respective boards of directors, the study was advertised in each local school; 68 (17.4%) female students met the inclusion/exclusion criteria and were further enrolled in the study (participants had 2.4±0.5 hours per week of exercise, twice a week within their physical education classes). Details on the participants' recruitment are in Figure 7.

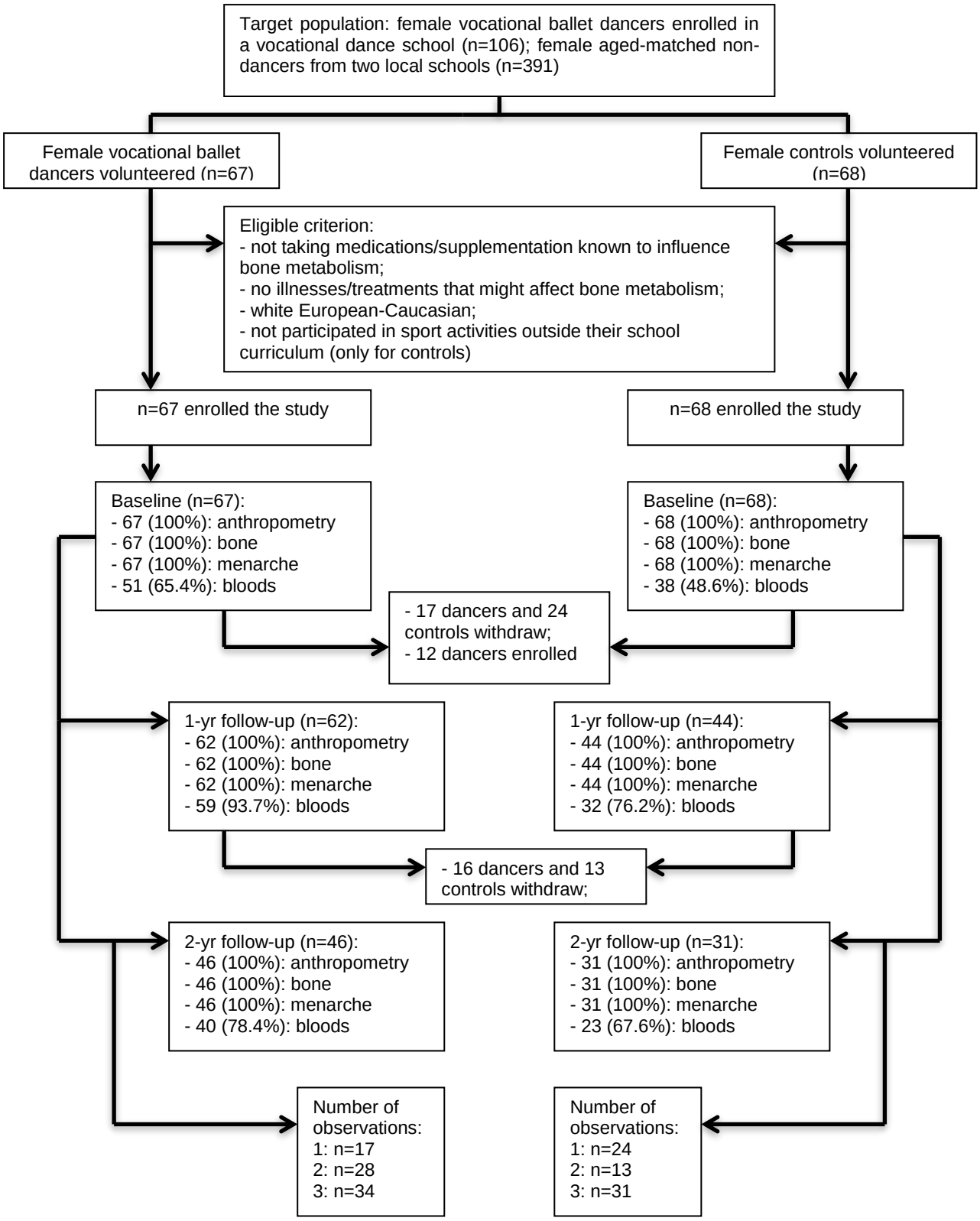


Figure 7. Study population flow.

All participants provided signed informed consent according to the Declaration of Helsinki. The study was approved by the ethics committee of the Regional Administration of Health of Lisbon, Portugal (Proc.063/CES/INV/2012).

Participants' measurements

Participants' enrolment (both dancers and controls) started at the year 2012 (September to November). Data were collected annually for three consecutive years, started at January 2013 and finished at March 2015. Annual collection occurred within the same period as the baseline measuring session. Specifically, information on bone mass, anthropometry, menarche and biological maturation were collect each January in vocational female dancers, whereas the same information was collected each March in controls. Bloods were collected each January in both groups.

Within the population of 67 vocational female dancers available for assessment at baseline (January 2013), 67 (100%) underwent anthropometric measures, participated in bone measurements and reported past/current menstrual; 51 (65.4%) donated blood. From 2013 to 2014, 12 vocational students were additionally recruited, while 17 withdraw the study due to professional dance training dropout or injuries. In accordance, in 2014, a total of 62 vocational students underwent anthropometric measures, participated in bone measurements and reported past/current menstrual; 59 (93.7%) donated blood. From 2014 to 2015, an additional of 16 vocational students withdraw the study for the reasons previously mentioned; a total of 46 vocational students were assessed in 2015 [all underwent anthropometric and bone measurements, menarche, and 40 (78.4%) donated blood].

Similarly, at baseline (March 2012), 68 (100%) controls underwent anthropometric measures, participated in bone measurements and reported menarche; 38 (48.6%) donated blood. From 2013 to 2014, only 44 controls were available for assessment (24 withdraw the study due to family relocation or lost of interest). All 44 (100%) participated in anthropometric, bone measurements and reported menarche; 32 (76.2%) donated blood. In 2015 an additional of 13 controls withdraw the study due to family relocation. From the 31 available for assessment in 2015, all underwent anthropometric, bone measurements and reported menarche; 23 (67.6%) donated blood.

After 3 years of data collection, 17 female vocational dance students were measured on one occasion (vs. 24 controls) and 62 (vs. 44 controls) on two or three occasions. Details on the participants' measurements appear in Figure 7.

Anthropometry, menarche and biological maturation assessment

Chronological age (obtained as decimal age) and anthropometry measurements were collected at one-year interval. Height, sitting height and body weight were measured in t-shirt, shorts and bare feet using a stadiometer (Seca, Seca217 portable stadiometer, Hamburg, Germany) with accuracy of 0.1 cm and an electric scale (TANITA BC-418 MA Segmental Body Composition Analyser; Tanita Corporation, Tokyo, Japan) with an accuracy of 0.1 kg. For each variable, two measurements were recorded in each evaluation; if the difference between them was greater than 0.3, a third measure was obtained. All measurements were administered by the same investigator every year.

At one-year interval, age of menarche was determined by questionnaire or email during the follow-up. At baseline, 27 female vocational dance students (40.3%) had reached the menarche (vs. 50 in controls – 73.5%). During the study, 16 vocational dance students (40%) (10 during the 1-yr and 6 during the 2-yr follow-up) reached de menarche (vs. 4 controls (22.2%) at the 1-yr and 2 at the 2-yr follow-up). Height, sitting height, weight and chronological age were assessed to estimate biological maturity using the offset equation [89]. Based on this equation, the year(s) to/from PHV and an estimation of the age at PHV were also predicted in all participants at one-year interval.

Hormonal analyses

Blood samples were collected in early morning after an 8-hour fasting. In menstruating subjects, samples were collected during the follicular phase of the menstrual cycle (fifth and ten days after the onset of menstrual bleeding). Blood samples were submitted to centrifugation at 2500g for 10 min; plasma and serum samples were stored at -80ºC until they were analysed.

Plasma oestrogens concentrations were measured by electrochemiluminescence immunoassay (ECLIA) kit (06656021190 Estradiol G3 Elecsys cobas and 100, Roche Diagnostic Systems); the intra-assay and inter-assay CV's were below or equal to 2.4% and 2.7%, respectively. Serum GH concentrations

were measured by an immunoradiometric assay kit (IRMA GH, ref. IM1397) from IMMUNOTECH SAS, (Prague, Czech Republic); the intra-assay and inter-assay CV's were below or equal to 2.7% and 7.1%, respectively. Serum IGF-1 concentrations were measured by an immunoradiometric assay kit (IRMA IGF-I, ref. A15729) from IMMUNOTECH SAS, (Marseille, France); the intra-assay and inter-assay CV's were below or equal to 6.3% and 6.8%, respectively.

Bone measurements

BMC (g) and BMD (g/cm^2) were determined for non-dominant forearm (33% radius), lumbar spine (L1-L4) (LS) and femoral neck (FN). Participants were assessed in two different centres using Dual-energy X-ray absorptiometry (DXA): Lunar (GE Lunar Prodigy) and Hologic (Discovery Wi). For consistency, the same certified technician performed all scans and analyses at both centres in each year.

Although previous studies have demonstrated a high correlation between Lunar and Hologic DXA BMD measurements [97–99], there is a tendency for Lunar model to inflate BMD values by 15% compared to Hologic [100]. Therefore, beside the daily calibration required from each DXA manufacturer, cross-calibration of the scanners was made using a group of 20 participants; the age of these 20 participants covered the age-range of the entire sample (both dancers and controls) used for the purpose of the present study. These participants were measured with both Lunar and Hologic within a period of 5 days. Regression equations using BMC and BMD from Lunar as dependent variable and BMC and BMD from Hologic as independent variable were performed from the data obtained from the participants included from cross-calibration. The correlation between the two DXA models were high (forearm BMD: r=0.96, adjusted r^2=0.93, std. error of estimate=0.03; LS BMD: r=0.96, adjusted r^2=0.92, std. error of estimate=0.05; FN BMD: r=0.97, adjusted r^2=0.93, std. error of estimate=0.05; forearm BMC: r=0.98, adjusted r^2=0.96, std. error of estimate=0.09; LS BMC: r=0.96, adjusted r^2=0.92, std. error of estimate=4.41; FN BMC: r=0.94, adjusted r^2=0.88, std. error of estimate=0.46). The Hologic BMC and BMD data were further converted to the Lunar data using the following equations: forearm BMD Lunar = -0,085263 + 1,356535*Hologic; LS BMD Lunar = 0,030762 + 1,161805*Hologic; FN BMD Lunar = 0,084782 + 1,116509*Hologic; forearm BMC Lunar = 0,148564 + 1,117715*Hologic; LS BMC Lunar = 7,143123 + 0,923483*Hologic; FN BMC Lunar = 0,079107+ 1,106219*Hologic.

Statistical analyses

Exploratory analyses were conducted in SPSS 20.0 software (IBM SPSS, Chicago, IL) to check for the presence of outliers; Mahalanobis test was used (2 dance students and 3 controls lied in an abnormal distance from other values and were excluded). Independent t-tests were used to compare general characteristics between dance population and controls at each measured occasion. Nonparametric tests (Mann–Whitney test) were applied if the data were not normally distributed; this was the case for the GH, IGF-1 and oestrogens. Repeated measures ANOVA were used to compare characteristics between the two groups across the measurement occasions. Based on a multilevel approach (hierarchical linear models) applied to longitudinal data, SuperMix software (SSI - Scientific Software International, Inc.) was used to investigate the predictors (i.e. age, body weight, height, menarche, oestrogen, GH and IGF-1) of bone mass accrual over time in each anatomical site, and to determine if the aforementioned factors could explain differences in terms of bone mass gains between VFD and matched-controls. These analyses are appropriate for study designs where data is organized in more than one level (in this case, participants are organized into groups); multilevel models can be used without the assumption of homogeneity that is required by ANCOVA. Chronological age was used as the metric of time: time 0 corresponds to mean chronological age (on average around 12 years of age); negative values at X axis represents the number of years before mean chronological age, whereas positive values represent number of years after mean chronological age. The level of significance was set at $p<0.05$.

RESULTS

General characteristics of the participants included in the follow-up are in Table 10. At the onset of the study, participants had a mean chronological age of approximately 12 years old (vocational dancers: 12.1±1.9; controls: 12.7±2.0, $p>0.05$). Over time vocational dancers always had a significantly lower body weight ($p<0.001$) and BMC/ BMD values at all anatomical sites ($p<0.01$/ $p<0.001$) compared to controls. Mean height differences were not significant throughout the follow-up ($p>0.05$). Vocational female dancers had their menarche approximately one year later than controls ($p<0.001$), but the estimated age at PHV did not significantly differ between groups (~12 years old for both groups). Serum IGF-1 concentrations were significantly higher in dance students compared to controls at 2yr follow-up ($p<0.001$).

Table 10. General characteristics of female dance students and aged- and sex-matched controls included in the follow-up

	Baseline		1yr Follow-up		2yrs Follow-up	
	DS	Controls	DS	Controls	DS	Controls
Age (yrs.)	12.1 ± 1.9	12.7 ± 2.0	13.1 ± 1.9	13.7 ± 2.0^{++}	14.1 ± 1.9^{++}	14.7 ± 2.0^{++}
Weight (kg)	35.2 ± 8.5**	51.9 ± 11.7	41.4 ± 8.1** $^{++}$	55.7 ± 11.3^{++}	44.2 ± 7.1** $^{++}$	58.5 ± 12.3^{+}
Height (cm)	147.1 ± 9.7	153.5 ± 9.2	153.9 ± 7.6^{++}	156.7 ± 8.3^{++}	157.0 ± 6.5^{++}	158.6 ± 6.9^{++}
Age menarche (yrs.)[(1)]	12.2 ± 1.9*	11.4 ± 1.2	-	-	-	-
Age at PHV (yrs.)[(1)]	12.5 ± 0.6	12.7 ± 0.9	-	-	-	-
Oestrogen (pg/mL)	32.9 ± 25.7	67.1 ± 107.2	54.1 ± 48.9	77.9 ± 90.3	52.1 ± 37.54	84.6 ± 81.7
GH (mIU/L)	5.5 ± 7.6	4.5 ± 4.4	5.3 ± 8.5	8.6 ± 10.1	8.9 ± 12.2	8.5 ± 7.9
IGF-1 (ng/mL)	291.6 ± 142.5	294.5 ± 124.4	350.7 ± 165.2	288.5 ± 130.0	371.1 ± 98.3*	258.7 ± 65.0
BMC FN (g)	3.18 ± 0.85*	3.86 ± 0.73	3.69 ± 0.85** $^{++}$	4.52 ± 0.74^{++}	3.86 ± 0.78** $^{+}$	4.64 ± 0.67^{+}
BMC LS (g)	33.8 ± 13.2**	46.9± 10.9	40.5 ± 12.7** $^{++}$	55.1 ± 11.0^{++}	45.0 ± 11.7** $^{++}$	57.4 ± 9.4^{++}
BMC FA (g)	1.3 ± 0.3**	1.8 ± 0.3	1.4 ± 0.3** $^{++}$	1.9 ± 0.3^{++}	1.6 ± 0.3** $^{++}$	2.0 ± 0.3
BMD FN (g/cm^2)	0.93 ± 0.17**	1.07 ± 0.13	0.94 ± 0.20** $^{++}$	1.07 ± 0.13	0.93 ± 0.20** $^{++}$	1.13 ± 0.20 $^{++}$
BMD LS (g/cm^2)	0.89 ± 0.17**	1.06 ± 0.16	0.91 ± 0.19** $^{++}$	1.15 ± 0.15 $^{++}$	0.93 ± 0.17** $^{++}$	1.21 ± 0.15 $^{+}$
BMD FA (g/cm^2)	0.59 ± 0.10**	0.75 ± 0.10	0.63 ± 0.10** $^{++}$	0.81 ± 0.08 $^{++}$	0.66 ± 0.10** $^{++}$	0.83 ± 0.08 $^{++}$

Values are means + SD

*p<0.05; **p<0.001; statistical different when comparing with controls

^{+}p<0.05; ^{++}p<0.001; statistical different when comparing with previous year measurement

DS: dance students; PHV: peak height velocity (estimation); BMC: bone mineral content; FN: femoral neck; LS: lumbar spine; FA: forearm; GH: growth hormone; IGF-1: insulin-like growth factor 1

[(1)] Means were calculated considered all dance students and controls assessed throughout the 3 years.

Table 11. Predictors of bone mass changes throughout the follow-up

	Estimates for dance students and controls					
Predictors	BMC FN	BMC LS	BMC FA	BMD FN	BMD LS	BMD FA
Intercept	4.041 ± 0.096	44.683 ± 1.186	1.519 ± 0.036	0.990 ± 0.022	0.946 ± 0.018	0.664 ± 0.012
Chronological age	0.106 ±0.033**	1.896 ± 0.407***	0.046 ± 0.012**	0.026 ± 0.008**	0.022 ± 0.005***	0.017 ± 0.004***
Chronological age^2	-0.022 ± 0.009*	-0.227 ± 0.096*	NS	-0.003 ± 0.001*	-0.002 ± 0.001*	-0.003 ± 0.0001**
Group	NS	3.937 ± 1.654*	0.333 ± 0.050***	0.095 ± 0.040*	0.158 ± 0.028***	0.129 ± 0.016***
Weight	0.033 ± 0.044***	0.424 ± 0.069***	0.007 ± 0.002**	0.004 ± 0.001	0.004 ± 0.001***	0.003 ± 0.001***
Height	NS	NS	NS	NS	NS	NS
Menarche	0.166 ± 0.085*	4.242 ± 0.947***	0.079 ± 0.029**	NS	0.042 ± 0.011***	NS
GH	NS	NS	NS	0.001 ± 0.0005*	NS	0.010 ± 0.0004**
IGF-1	NS	NS	0.0003 ±0.0001**	NS	NS	0.0001 ± 0.00004*
Oestrogen	NS	NS	NS	NS	NS	NS
Chronological age*Group	NS	NS	0.039 ± 0.016*	0.002 ± 0.002*	0.025 ± 0.006***	0.012 ± 0.005*
Weight*Group	NS	NS	-0.012 ± 0.004**	NS	NS	-0.003 ± 0.001*
Menarche*Group	NS	NS	NS	-	NS	-
GH*Group	-	-	-	NS	-	NS
IGF-1*Group	-	-	NS	-	-	NS
Oestrogen*Group	-	-	-	-	-	-

Multilevel models were conducted
Values are estimates + standard errors
* p<0.05; ** p<0.01; *** p<0.001; significant predictor
NS: not significant
BMC: bone mineral content; BMD: bone mineral density; FN: femoral neck; LS: lumbar spine; FA: forearm; GH: growth hormone; IGF-1: insulin-like growth factor 1

The coefficients that a) predict bone mass changes over time and b) identify potential factors that might explain differences in BMC/ BMD between groups (variable*group) are summarized in Table 11. The interaction between groups for BMC gains (chronological age*group) was not significant at impact sites (FN and LS; $p>0.05$); vocational dance students always revealed lower BMC values at these anatomical sites than controls during the follow-up. On the other hand, considering BMC at the forearm (non-impact site) and BMD at all the other anatomical sites, the interaction chronological age*group was significantly positive (BMC forearm: $\beta=0.039$, $p<0.05$; BMD FN: $\beta=0.002$, $p<0.05$; BMD LS: $\beta=0.025$, $p<0.001$; BMD forearm: $\beta=0.012$, $p<0.05$). Menarche was found to be a significant predictor of bone mass gains: participants that had their first menstruation had significant higher BMC at the FN ($\beta=0.166$, $p<0.01$), LS ($\beta=4.242$, $p<0.001$) and forearm ($\beta=0.079$, $p<0.01$) compared to the ones that have not reached menarche. Body weight also significantly predicted bone mass accruals throughout the follow-up at all anatomical sites (BMC FN: $\beta=0.033$, $p<0.001$; BMC/ BMD LS: $\beta=0.424$, $p<0.001$/ $\beta=0.004$, $p<0.001$; BMC/ BMD forearm: $\beta=0.07$, $p<0.001$/ $\beta=0.003$, $p<0.001$). Serum concentrations of GH and IGF-1 were also found to be significant predictors of BMC and BMD changes over time at the forearm: as serum concentrations of these hormones increase, BMC and BMD values at this anatomical site also increased ($\beta=0.0003$, $p<0.001$; $\beta=0.0001$, $p<0.05$ respectively). GH also had a significant predictive effect on BMD at the FN ($\beta=0.001$, $p<0.05$). Nevertheless, when a group interaction were analysed, it was not found a significant interaction at impact sites (both FN and LS) between menarche*group and weight*group; these variables (menarche and body weight) did not explain group differences at the FN and LS. However, a significant group effect was found at the non-impacted site (forearm), indicating that BMC and BMD accruals differ between groups according to body weight: female vocational dance students will have lower bone mass values at the forearm (BMC: 0.012g lower, $p<0.01$; BMD: 0.003g lower, $p<0.05$) if their body weight is lower than controls of the same age and height. It was not found a significant interaction between GH and IGF-1 with group (GH*Group; IGH-1*Group), meaning that GH and IGF-1 concentrations did not explain group differences seen on bone mass gains at the forearm when comparing vocational dancers with controls.

DISCUSSION

The mechanisms responsible for low BMD phenotypes in general population are well documented, but evidence regarding athletic populations is lacking. In elite dancing, for instance, it has been recently shown that both professional and vocational dance students have increased odds for low BMD compared to non-dancers [data not published]; however, the associated factors are not completely clear [109]. To the best of my knowledge, this is the first longitudinal study aiming to investigate bone mass accruals and its association with circulating levels of oestrogens, GH and IGF-1 in VFD. The main finding was the low BMC and BMD values displayed by dance students at baseline and the absence of a “catch-up” accrual during the follow-up in relation to controls; dancers´ bone mass values remained consistently lower compared to non-exercising controls as they progressed on their professional training. Apparently, endocrine mechanisms seem not to be able to explain differences between female vocational dancers and non-exercising controls in terms of bone mass gains.

Bone mass accruals significantly increased during the follow-up in both groups, as well as circulating levels of oestrogens, GH and IGF-1. This was expected since our population is growing [3,117]. Clinical observations claim that growing bone is more responsive to mechanical loading than mature bone [149]; during puberty, osteogenic hormones are likely to interact with physical exercise to positively affect bone mass accruals [149]. Considering our results, at 2-yr follow-up, IGF-1 serum concentrations are significantly increased in our vocational dancers compared to controls. These increments might reflect a dance training effect since higher circulating levels of serum IGF-1 has been documented in young athletes via GH-independent mechanism [140,150], leading further to greater bone mass accruals compared to non-exercise participants. However, in our dance population the higher increments in IGF-1 serum levels seem not to be translated into higher bone mass gains. Indeed, it would have been expected that bone mass differences between groups have lessen during the follow-up (particularly at impact sites), not only due to increasing levels of circulating IGF-1, but also due to effects of dance training on bone cells [87,132]; this was not observed. The present study cannot explain these findings. It seems unlikely that group differences were caused by delayed growth/ maturity, as age at PHV and GH/oestrogens concentrations did not differ between groups; also, body weight was a significant predictor of bone mass differences

between groups only at the forearm. Instead, I suggest that skeletal biological determinants might be involved as the dynamic actions of liver-derived IGF-1 in bone involve complex signalling pathways that might directly affect both osteoblasts and osteoclasts [141]. IGF-1 can either act on the commitment of mesenchymal stem cells to oesteoprogenitor cells [151], or induce RANKL synthesis in osteoclasts, leading to osteoclastogenesis [140]. Furthermore, it has been hypothesised that changes in IGF-1 receptors in osteoclasts number or affinity could determine an increase or decrease in bone formation [142]. Whether the aforementioned factors explain the low BMC and BMD values seen in our dancers warrant further investigation.

Bone mass formation and development are influenced by genetic and endocrine mechanisms that are modulated by environmental factors such as physical exercise [152,153]. This means that the degree to which physical exercise influences bone formation depends on individual's genetic background [153–155], and bone specific characteristics (i.e. cortical or trabecular; weight-bearing or non weight-bearing) [133,156]. The fact that GH and IGF-1 serum concentrations was only significantly associated with bone mass values at non-impact sites (forearm), might indicate that gene-environment factors are interacting differently in determining dancers' bone mass phenotypes across skeletal sites. Moreover, the fact that body weight, a well-known risk factor for low bone mineralization, was found to be a significant predictor of group differences in terms of bone mass gains at forearm, but not at impact sites, further supports the aforementioned hypothesis (as well as the absence of a "catch-up" bone mass accrual by dancers in relation to controls at the FN and LS). This reinforces the view that other factors apart from osteoporosis traditional risk factors seem to be major determinants of bone mass acquisition of young vocational dancers. Other studies on athletic populations also found that adolescent runners with low bone mass values at baseline continued to display low bone parameters after 3-yr follow-up [157]. The determinants of bone mass accruals in athletic populations may be different of the general population due to specific sport adaptations and body characteristics previously selected for the profession. Therefore, taken together, future studies on dancers (and on other elite athletes) should consider genetic markers as well as gene-environment interactions to further understand the pathogenesis of low bone mass parameters, particularly at weight-bearing sites.

Although this is the first study measuring longitudinally bone mass accruals and bone osteogenic hormones in vocational dance students, our findings should be interpreted considering some limitations. Present data is observational and cause-effect cannot be determined. Our sample of vocational dancers is large and well-defined considering the entire population of elite dance students performing at a national level (response rate to the study was high – 63.2%); nevertheless, for a mixed-longitudinal design the sample size is relatively small. Furthermore, as adolescents, our sample is going through a critical period characterised by significant biological changes. This fact might explain the high variance seen in terms of hormonal values in both groups, which reinforces the need for future studies to consider larger sample sizes. The main outcome in this study – BMC, measured by DXA - does not account for changes in bone microarchitecture; however, this device (as well as BMC as main outcome) is considered the “gold standard” to longitudinally measure children [158].

CONCLUSIONS

Female vocational dance students have low bone mass values compared to matched-controls at both impact and non-impact sites. As these adolescents progress on their professional training, their bone mineral content remains consistently lower compared to non-exercising controls. Endocrine mechanisms seem not to explain the differences in terms of bone mass gains between groups.

CHAPTER 8: ASSOCIATIONS BETWEEN NUTRITION, ENERGY EXPENDITURE, AND ENERGY AVAILABILITY WITH BONE MASS ACQUISITION IN FEMALE AND MALE VOCATIONAL DANCE STUDENTS: A MIXED-LONGITUDINAL STUDY (STUDY 4)

Parts of this chapter have been submitted in the peer-reviewed journal *Bone*. The author of this Thesis appears as the leading author.

ABSTRACT

Aim To determine a) whether nutrition, energy expenditure and energy availability are significant predictors of BMC and BMD changes during growth in female and male vocational dance students, and b) if these factors are able to explain difference in bone mass accruals between vocational dancers (female and male) and controls.

Methods Sixty-three female vocational dancers and 50 aged- and sex- matched controls (12.8±2.2yrs and 13.0±2.1yrs at baseline, respectively), and 38 male vocational dancers and 68 aged- and sex- matched controls (12.7±2.2yrs and 13.0±1.8yrs at baseline, respectively) were monitored for three years. BMC and BMD were measured annually at impact sites (FN and LS), and non-impact site (forearm) using DXA. Anthropometry, age at menarche (questionnaire), nutrition (3-day record), energy expenditure (accelerometer), and IGF-1 serum concentration (immunoradiometric assays) were also measured for the same period.

Results Female and male vocational dancers had consistently significantly reduced body weight ($p<0.001$) and bone mass values at all anatomical sites ($p<0.001$) than controls. Serum IGF-1 concentrations did not differ between male vocational dancers and controls. At baseline, calcium intake was significantly increased in female vocational dancers compared to controls ($p<0.05$). Male vocational dancers' fat and carbohydrate intakes were significantly lower than matched controls ($p<0.001$ and $p<0.05$, respectively). Energy availability of both female and male vocational dancers lied within the normal range (i.e. <30 kcal/kgFFM/day). A significant group effect was found at the FN regarding energy intake ($p<0.05$). No significant predictors were found to explain bone mass differences between groups in males.

Conclusion Energy intake and energy availability were found to positively predict bone mass gains at the FN (impact site) in female dancers. Nutrition, energy expenditure and energy availability did not explain the lower bone mass values revealed by male dancers compared to controls at both impact and non-impact sites.

KEYWORDS BMC; BMD; follow-up; ballet; athletic population; female athlete triad

INTRODUCTION

Osteoporosis is a major public health concern [1]. This disease is characterised by decreased BMD and skeletal fragility, leading to increased fracture risk and immobility, which might affect people's quality of life [2].

Exercise can be one mean of preventing low BMD [1]. Reports on short-term interventions in healthy participants show that weight-bearing exercise provide protection against the risk of low BMD [1,10]. In accordance, the World Health Organisation provides physical exercise guidelines to promote bone health [159]. Nevertheless, while leisure exercise can prevent low BMD, the effects of sport training on bone health are still controversial [11,12]. For instance, some participants (particularly females) involved in elite sports have lower BMD values than their non-exercising counterparts [123,160–162]. It is general believed that the low BMD in these female participants involves mechanisms related to the GH– IGF-I axis and HHG axis [163], wherein the presence of low body weight and menstrual disturbances due to intensive training is assumed to modulate the aforementioned axis, leading to an alteration in the hypothalamic pituitary [11,20–23]; which further impairs bone mineralization. This phenomenon is known as the "female athlete triad" [11]. Recently, this concept has been expanded to RED-S', i.e. relative energy deficiency in sport [24]. Indeed, it has been suggested that the main factor that triggers the triad is an energy deficiency in relation to the balance between energy intake and energy expenditure, which further impairs GH– IGF-I and HHG axis and, consequently, bone health [24]. In accordance, the concept of relative energy deficiency for performance may also affect men [24].

As an aesthetic activity, elite dancers are exposed to high levels of artistic and physical fitness demands, whereas low body weight is strongly emphasised [13]. In accordance, to correspond with the aesthetic demands, elite dancers may restrict their energy intake, leading to a negative energy balance [17–19]. This energy imbalance can affect the hypothalamus, decreasing the circulating levels of IGF-1. Indeed, it has been reported that these hormones are sensitive to changes in nutrition intake, particularly caloric intake, hindering further bone mineralization [140]. Nevertheless, in the previous chapter of the current Thesis, female vocational dance students had lower BMD compared to controls, although their IGF-1 serum concentrations was not different from controls. This suggests that energy intake might not be playing a significant role in determining dancers' BMD phenotypes.

Therefore, the aims of the present study were to determine a) whether nutrition, energy expenditure and energy availability are significant predictors of BMC and BMD changes throughout growth in female and male vocational dance students, and b) if these factors are able to explain difference in bone mass accruals between vocational dancers (female and male) and matched controls.

METHODS

Participants' recruitment

The participants' recruitment process was done as described in the previous chapter (Chapter 7); however, for this study it was also recruited male vocational dance students. From a total of 74 male dance students that were enrolled at the vocational school in the academic year 2012/2013, 38 (51.4%) volunteered. All of these completed a questionnaire concerning their ethnicity, physical activity, medical history, and past/current calcium/vitamin D supplementation. As none of the volunteers reported consumption of medications/supplementation known to influence bone metabolism, nor reported illnesses/treatments that might affect bone metabolism, all 38 were enrolled in the study [all described themselves as white European-Caucasian, and were involved in 19.5±7.2 hours of dance training per week during 5 to 6 week days].

Out of the 67 female vocational dance students included in the previous study (Study 7), 63 were included in the present study.

Male vocational dance students and adolescent non-dancers were also recruited from two random local state schools to act as controls. The same recruitment process as described on the previous study (Study 7) was used for male controls; 68 (8.0%) male students met the inclusion/exclusion criteria and were further enrolled in the study (participants had 2.4±0.5 hours per week of exercise, twice a week within their physical education classes). Regarding controls for the 63 female vocational dance students, out of the 68 recruited in the previous study (Study 7), 50 enrolled the present study.

All participants provided signed informed consent according to the Declaration of Helsinki. The study was approved by the ethics committee of the Regional Administration of Health of Lisbon, Portugal (Proc.063/CES/INV/2012).

Participants' measurements

As previously described (Study 7), participants' enrolment (both dancers and controls) started at the year 2012 (September to November). Data were collected annually for three consecutive years, starting at January 2013 and finishing at March 2015. Annual collection occurred within the same period as the baseline measurements. Information on bone mass, anthropometry, menstruation biological maturation [estimation of the age at PHV)], nutrition (energy intake, calcium, fat, carbohydrates) and energy availability were collect each January for vocational dance students, whereas the same information was collected each March in controls. Bloods were collected each January in both groups. Information on physical exercise and bone specific loading was collected on the last year of measurements.

Within the population of 63 female dance students (vs. 38 male vocational dance students) available for assessment at baseline (January 2013), 63 (38 male) (100%) underwent anthropometric measures, participated in bone measurements and reported past/current menstrual (only female). Sixty-one female (vs. 33 male) (96.8% and 86.8, respectively) reported nutrition and 54 female (vs. 34 male) (85.7% and 89.5, respectively) were assessed for energy expenditure; 51 female (28 male) (80.9% and 73.7%, respectively) donated blood. From 2013 to 2014, 12 female dance students were additionally recruited, while 17 female and 5 withdraw the study due to professional dance training dropout or illness. In accordance, in 2014, a total of 58 female dance students (vs. 33 male) underwent anthropometric measures, participated in bone measurements and reported past/current menstrual. Forty-nine female (vs. 29 male) (84.5% and 87.9%, respectively) reported nutrition intakes and 50 female (vs. 26 male) (86.2% and 78.8%, respectively) were assessed for energy expenditure; 54 female and 27 male (93.1% and 81.8%, respectively) donated blood. From 2014 to 2015, an additional of 16 female dance students and 12 male vocational dancers withdraw the study for the reasons previously mentioned. A total of 42 female dance students (vs. 21 male) were assessed in 2015 [all underwent anthropometric and bone measurements, reported past/current menstrual, 30 female (vs. 16 male) (71.4% and 76.2%, respectively) reported nutrition intakes, 36 female (vs. 12 male) (85.7% and 57.1%, respectively) were assessed for energy expenditure; 40 female (vs. 13 male) (95.2% and 61.9%, respectively) donated blood]. Similarly, at baseline (March 2012), 50 female controls (vs. 47 male) (100%) controls underwent anthropometric measures, participated in bone measurements,

reported past/current menstrual (female) and 46 female (vs. 34 male) (92.0% and 72.3%, respectively) reported nutrition; 38 female (vs. 36 male) (76.0% and 76.6%, respectively) donated blood. From 2013 to 2014, only 44 female and 40 male controls were available for assessment (24 female and 7 male withdraw the study due to family relocation or lost of interest). All 44 and 40 (100%) participated in anthropometric, bone measurements and reported past/current menstrual. Thirty-five female (vs. 27 male) (79.5% and 67.5%, respectively) reported nutrition; 32 female (vs. 29 male) (79.5% and 72.5%, respectively) donated blood. In 2015 an additional of 13 female and 11 male controls withdraw the study due to family relocation. From the 31 and 29 controls available for assessment in 2015, all underwent anthropometric, bone measurements and reported past/current menstrual. A total of 28 female (vs. 25 male) (90.3% and 86.2%, respectively) reported nutrition; 23 female (vs. 19 male) (74.3% and 65.5%, respectively) donated blood.

Anthropometry, menarche and biological maturation assessment

The same procedures as the previous study (Study 3) were followed.

Nutritional intake and energy availability

Nutrient intakes were recorded via a 3-day food diary, previously validated [90]. Participants were asked to record all food and beverages consumed during two school days and one weekend day following appropriate instructions. The software Food Processor SQL Edition, version 9.8.1. was used to estimate average energy and nutrition intakes. During the same week that nutrition information was collected, energy expenditure was also estimated using an accelerometer – SenseWear [91]; each participant used the device for 7 consecutive days. Energy availability was estimated using standard protocols (http://www.femaleathletetriad.org/calculators/); information on dietary energy intake (provided by the food diary), exercise energy expenditure (information retrieved from the accelerometer), and body fat percentage (measured by DXA) was used for the estimation of energy availability.

Hormonal analyses

Blood samples were collected in early morning after an 8-hour fasting. Blood samples were submitted to centrifugation at 2500g for 10 min; plasma and serum samples were stored at -80ºC until they were analysed. In male (both dancers and

controls), serum IGF-1 concentrations were measured by an immunoradiometric assay kit (IRMA IGF-I, ref. A15729) from IMMUNOTECH SAS, (Marseille, France); the intra-assay and inter-assay CV's were below or equal to 6.3% and 6.8%, respectively. IGF-1 data for female vocational dancers and female controls were used from the previous study (Chapter 7).

Physical exercise in terms of bone specific loading

The BPAQ was administrated to all participants included in the longitudinal analysis (data collected on the last year of measurements – 2015). Participants were asked to record all regular physical exercise activities performed throughout their life, including years and frequency (times per week) of participation. A score for physical exercise in terms of bone specific loading was further derived for each participant. The BPAQ has been described in detail previous [92]. Briefly, the BPAQ has been designed to capture bone-relevant weight-bearing exercise history (an algorithm was created to account the factors of load intensity, years of participation, and frequency of current and historical physical exercise) [92]. It has been reported that the BPAQ score is positively associated with DXA BMD outcomes [92]. The BPAQ was originally validated for young adults, however, it can also be applied to children (a specific age weighting factor in the algorithm is applied) [92]. In accordance, several reports have been using BPAQ in children and adolescents to predict bone-relevant weight-bearing exercise [93–95]. Our control participants have not been involved in methodical exercise (exclusion criteria included those who participated or had previously participated in methodical physical exercise activities outside school curriculum). In order to account for the physical education lessons at school (twice a week) it was assumed the following activities for control participants: walking/hiking, running/jogging, soccer and jump rope.

Bone measurements

The same procedures as the previous study (Chapter 7) were followed.

Statistical analyses

Exploratory analyses were conducted in SPSS 20.0 software (IBM SPSS, Chicago, IL) to check for the presence of outliers; Mahalanobis test was used (BMD from 2

dance students and 3 controls lied in an abnormal distance from other values and were excluded).

Independent t-tests were used to compare general characteristics between pre-professional dancers and controls at each measured occasion (stratified by sex). Based on a multilevel approach applied to longitudinal data, SuperMix software (SSI - Scientific Software International, Inc.) was used to investigate the predictors of bone mass accrual over time in each anatomical site. These analyses are appropriate for study designs where data is organized in more than one level (in this case, participants are organized into groups); multilevel models can be used without the assumption of homogeneity that is required by ANCOVA. Chronological age was used as the metric of time: time 0 corresponds to mean chronological age (on average around 12 years of age); negative values at X axis represents the number of years before mean chronological age, whereas positive values represent number of years after mean chronological age.

RESULTS

General characteristics of the participants included in the follow-up are in Table 12. At the onset of the study, both female and male participants had a mean chronological age of approximately 12 years old (female dancers: 12.8±2.2, female controls: 13.0±2.1, $p>0.05$; male dancers: 12.7±2.2, male controls: 12.7±2.2, $p>0.05$). Over time female and male vocational dancers always had a significantly lower body weight ($p<0.001$) compared to matched controls. Female vocational dancers had their menarche approximately one year later than controls ($p<0.001$), but the estimated age at PHV did not differ between groups (~12 years old for both groups). In turn, the age at PHV in male vocational dancers was significantly later than their controls (~6 months later, $p<0.001$). At baseline, calcium intake was significantly increased in female vocational dancers compared to controls ($p<0.05$), but there was no difference between groups throughout the follow-up. Male vocational dancers' fat and carbohydrate intakes were significantly lower than matched controls ($p<0.001$ and $p<0.05$, respectively). Throughout the follow-up, energy availability of both female and male vocational dancers was within the normal range (i.e. <30 kcal/kgFFM/day). Serum IGF-1 concentrations were significantly higher in female dance students compared to controls at 2yr follow-up ($p<0.001$); though their male counterparts reported no significant difference. The amount of

current and past physical exercise relevant to the skeleton was significantly higher in female vocational dancers compared with controls: score of 67.7±11.7 vs. 3.8±3.7, $p<0.001$ (current exercise), and 20.7±11.4 vs. 1.3±1.5, $p<0.001$ (past exercise), respectively. The same was revealed by male dancers compared with their controls: score of 74.1±3.8 vs. 4.3 ± 2.3, $p<0.001$ (current exercise), and 22.3 ± 5.0 vs. 2.1±0.9, $p<0.001$ (past exercise), respectively.

Table 12. General characteristics of the female and male vocational dance students and aged- and sex-matched controls included in the follow-up

	Female vocational dance students					
	Baseline		1yr Follow-up		2yrs Follow-up	
	VD	Controls	VD	Controls	VD	Controls
Age (yrs.)	12.8 ± 2.2	13.0 ± 2.1	13.6 ± 2.0^{+++}	14.0 ± 2.1^{+++}	14.9 ± 1.9^{+++}	15.0 ± 2.0^{+++}
Weight (kg)	39.3 ± 9.0***	52.7 ± 12.8	43.1 ± 8.1*** $^{+++}$	55.2 ± 10.7^{+++}	44.5 ± 6.7*** $^{+++}$	58.6 ± 12.4^{++}
Height (cm)	151.7 ± 10.5	154.6 ± 8.8	154.5 ± 8.1^{+++}	156.8 ± 8.1^{+++}	156.9 ± 6.6^{+++}	158.4 ± 6.9
Age menarche (yrs.)[(1)]	12.5 ± 1.4	11.5 ± 1.2	-	-	-	-
Age at PHV (yrs.)[(1)]	12.6 ± 1.2	12.6 ± 0.8	-	-	-	-
Energy Intake (Kcal/day)	1724.5 ± 508.0	1855.3 ± 479.2	1787.75 ± 444.98	1824.53 ± 674.76	1722.5 ± 412.0	1673.4 ± 460.0
Calcium (mg)	827.2 ± 360.2*	652.33 ± 332.0	871.8 ± 487.1	718.9 ± 316.1	758.71 ± 297.3	756.0 ± 234.9
Carbohydrates (g)	224.6 ± 69.9	238.8 ± 85.5	222.77 ± 58.59	229.23 ± 99.95	206.7 ± 48.49^{+}	274.4 ± 346.1
Fat (g)	59.1 ± 83.7*	83.7 ± 46.9	19.68 ± 2.81	25.75 ± 4.70	61.8 ± 20.4	55.5 ± 19.3^{+}
Energy availability (kcal/kgFFM/day)	46.9 ± 22.9	-	39.3 ± 18.8^{+++}	-	31.0 ± 13.1	-
IGF-1 (ng/mL)	304.4 ± 120.7	314.5 ± 115.7	329.21 ± 152.05	298.00 ± 111.09	334.5 ± 104.7*	261.7 ± 63.3
Bone specific loading (current)	-	-	-	-	67.7 ± 11.7***	3.8 ± 3.7
Bone specific loading (past)	-	-	-	-	20.7 ± 11.4***	1.3 ± 1.5
BMC FN (g)	3.54 ± 0.95**	3.97 ± 0.89	3.73 ± 0.90*** $^{+++}$	4.51 ± 0.71^{+++}	3.76 ± 0.77*** $^{++}$	4.65 ± 0.68^{+}
BMC LS (g)	38.53 ± 13.8***	48.77 ± 12.85	41.67 ± 13.21*** $^{+++}$	54.38 ± 10.38^{+++}	43.76 ± 11.94*** $^{+++}$	57.23 ± 9.31^{++}
BMC FA (g)	1.37 ± 0.31***	1.84 ± 0.33	1.46 ± 0.31*** $^{+++}$	1.92 ± 0.28^{+++}	1.52 ± 0.29*** $^{+++}$	1.95 ± 0.27
BMD FN (g/cm^2)	0.93 ± 0.17***	1.08 ± 0.14	0.94 ± 0.19** $^{+++}$	1.06 ± 0.13	0.93 ± 0.20***	1.13 ± 0.21^{+}
BMD LS (g/cm^2)	0.90 ± 0.17***	1.07 ± 0.18	0.91 ± 0.19*** $^{+++}$	1.14 ± 0.14^{+++}	0.93 ± 0.17***	1.20 ± 0.15^{++}
BMD FA (g/cm^2)	0.60 ± 0.09***	0.76 ± 0.10	0.63 ± 0.10*** $^{+++}$	0.81 ± 0.08^{+++}	0.66 ± 0.10*** $^{+++}$	0.84 ± 0.08^{+++}
	Male vocational dance students					
	Baseline		1yr Follow-up		2yrs Follow-up	
	VD	Controls	VD	Controls	VD	Controls
Age (yrs.)	12.7 ± 2.2	13.0 ± 1.8	13.7 ± 2.1^{+++}	13.9 ± 1.7 $^{+++}$	14.8 ± 2.2^{+++}	14.7 ± 1.6^{+++}
Weight (kg)	44.6 ± 13.5**	54.8 ± 13.2	45.7 ± 9.2*** $^{+++}$	59.7 ± 13.5 $^{+++}$	49.2 ± 11.0* $^{++}$	59.9 ± 12.5^{+++}
Height (cm)	156.6 ± 15.8	159.8 ± 10.3	160.5 ± 11.3 $^{++}$	163.5 ± 9.7 $^{+++}$	163.2 ± 9.7^{++}	165.7 ± 8.2^{+++}
Age at PHV (yrs.)[(1)]	12.7 ± 1.0***	12.2 ± 0.8	-	-	-	-
Energy Intake (Kcal/day)	1599.3 ± 463.9	1900.0 ± 451.8	1698.7 ± 396.6	2080.5 ± 467.6	1900.3 ± 280.2	1880.3 ± 455.3
Calcium (mg)	725.3 ± 370.0	645.82 ± 214.59	808.3 ± 334.6	866.6 ± 284.0 $^{+}$	714.8 ± 212.9	850.0 ± 405.5^{+}
Carbohydrates (g)	211.2 ± 68.5*	232.8 ± 84.2	213.0 ± 54.2	253.4 ± 81.9	232.9 ± 27.8	224.5 ± 74.76
Fat (g)	54.60 ± 19.23***	82.39 ± 22.77	60.2 ± 17.4	70.6 ± 19.3	71.1 ± 10.9	61.3 ± 17.5
Energy availability (kcal/kgFFM/day)	36.2 ± 18.1	-	29.2 ± 11.7	-	26.9 ± 13.0	-
IGF-1 (ng/mL)	257.8 ± 71.5	241.8 ± 73.7	272.7 ± 97.2^{+}	266.0 ± 106.7	281.5 ± 96.9	321.5 ± 125.5
Bone specific loading (current)	-	-	-	-	74.1 ± 3.8***	4.3 ± 2.3
Bone specific loading (past)	-	-	-	-	22.3 ± 5.0***	2.1 ± 0.9
BMC FN (g)	3.93 ± 1.32*	4.55 ± 0.98	4.06 ± 1.01*** $^{+++}$	5.14 ± 1.20^{+++}	4.56 ± 1.39^{++}	5.33 ± 1.28^{+++}
BMC LS (g)	39.97 ± 19.16*	47.07 ± 12.43	41.75 ± 17.76** $^{+++}$	54.34 ± 15.38^{+++}	46.24 ± 10.06^{+}	56.75 ± 15.87^{+++}
BMC FA (g)	2.25 ± 0.98*	1.83 ± 0.33	1.53 ± 0.36*** $^{+++}$	2.06 ± 0.40^{+++}	1.78 ± 0.60	2.11 ± 0.44^{+++}
BMD FN (g/cm^2)	0.90 ± 0.23***	1.13 ± 0.18	0.91 ± 0.20** $^{+++}$	1.07 ± 0.25^{+}	1.10 ± 0.23	1.14 ± 0.18^{+}
BMD LS (g/cm^2)	0.82 ± 0.21***	0.98 ± 0.18	0.84 ± 0.20*** $^{+++}$	1.05 ± 0.21^{+++}	0.95 ± 0.24	1.04 ± 0.21^{+++}
BMD FA (g/cm^2)	0.59 ± 0.10***	0.72 ± 0.10	0.62 ± 0.09*** $^{+++}$	0.80 ± 0.12^{+++}	0.67 ± 0.12**$^{++}$	0.81 ± 0.11

Values are means ± SD

* p<0.05; ** p<0.01; *** p<0.001; statistical significant differences between groups

+ p<0.05; ** p<0.01; *** p<0.001; statistical significant differences within groups

VD: vocational dance students; PHV: peak height velocity (estimation); BMC: bone mineral content; BMD: bone mineral density; FN: femoral neck; LS: lumbar spine; FA: forearm; GH: growth hormone; IGF-1: insulin-like growth factor 1

[(1)] Means were calculated considering all pre-professional ballet dancers and controls assessed throughout the 3 years.

Bone mass accruals over time are displayed in Table 13. In terms of BMC gains in female, the interaction between group and age (i.e. chronological age*group) was not significant (p>0.05), meaning that female vocational dancers always revealed lower BMC values at all anatomical sites than controls during the follow-up. This was also the case for BMD at the forearm (p>0.05). On the other hand, the interaction chronological age*group for BMD at the FN and LS was significantly positive (BMD FN: 0.02, p<0.01; BMD LS: 0.02, p<0.01), meaning that baseline differences between groups in terms of bone mass values have been accentuated during the follow-up at these anatomical sites. The only significant interaction found in male participants (chronological age*group) was at the FN (BMC: 0.24, p<0.05).

The coefficients that a) predict bone mass changes over time and b) identify potential factors that might explain differences in BMC/ BMD between groups (variable*group) are summarized in Table 13. In female vocational dance students, the associations between body weight/ menarche with bone mass accruals at different anatomical sites have been reported in the previous study (Study 3) and will not be reported here. In the present study though it was found that energy intake could explain differences in terms of bone mass gains between female dancers and controls at the FN. Indeed, a significant group effect was found at the FN regarding energy intake in female dancers (i.e. energy intake*group): female vocational dance students will have higher bone mass values at this site (0.00004 higher, p<0.05) if their energy is higher compared to controls of the same age and height. Nevertheless, it was not found predictors that could explain the lower BMC values displayed by female dancers compared with controls at the LS and forearm. Considering male vocational dance students, body weight was a significant predictor of bone mass accruals throughout the follow-up at the FN and LS (BMC FN: β=0.04, p<0.05; BMC LS: β=0.44, p<0.05; BMD). However, the present study was not able to find significant predictors able to explain bone mass differences between groups (i.e. male dancer vs. controls).

Table 13. Predictors of bone mass changes throughout the follow-up in vocational dance students

	Estimates for female vocational dance students and controls					
Predictors	BMC FN	BMC LS	BMC FA	BMD FN	BMD LS	BMD FA
Intercept	3.77 ± 0.10	44.77 ± 1.21***	1.52 ± 0.04	0.65 ± 0.01***	0.92 ± 0.02***	0.95 ± 0.03***
Chronological age	0.28 ± 0.03***	2.00 ± 0.44***	0.01 ± 0.01***	0.01 ± 0.004**	0.02 ± 0.01***	NS
Chronological age^2	-0.03 ± 0.001***	NS	NS	-0.02 ± 0.001**	-0.002 ± 0.001*	NS
Group	0.25 ± 0.13*	0.99 ± 1.70***	0.26 ± 0.06***	0.13 ± 0.02***	0.12 ± 0.03***	0.10 ± 0.03**
Energy intake	NS	NS	NS	-0.0001± 0.00001**	NS	NS
Calcium	NS	NS	NS	NS	NS	NS
Carbohydrates	NS	NS	NS	NS	NS	NS
Fat	NS	NS	NS	NS	NS	NS
Energy availability	0.01 ± 0.003*	NS	NS	NS	NS	NS
Chronological age*Group	NS	NS	NS	0.02 ± 0.001**	0.02 ± 0.01**	NS
Energy intake*Group	-	-	-	0.00004 ± 0.0002*	-	-
Calcium*Group	-	-	-	-	-	-
Carbohydrates*Group	-	-	-	-	-	-
Fat*Group	-	-	-	-	-	-
	Estimates for male vocational dance students and controls					
Predictors	BMC FN	BMC LS	BMC FA	BMD FN	BMD LS	BMD FA
Intercept	4.13 ± 0.18	42.06 ± 1.98***	1.97 ± 0.09***	0.89 ± 0.04***	0.85 ± 0.03***	0.60 ± 0.01**
Chronological age	NS	2.28 ± 0.95*	NS	NS	NS	0.02 ± 0.01**
Chronological age^2	0.05 ± 0.01***	0.95 ± 0.16***	NS	0.01 ± 0.004**	0.01 ± 0.002***	0.05 ± 0.01**
Group	0.48 ± 0.20*	5.52 ± 2.24*	NS	0.18 ± 0.05***	0.12 ± 0.03***	0.14 ± 0.02***
Weight	0.04 ± 0.02*	0.44 ± 0.22*	NS	NS	NS	NS
Height	NS	0.41 ± 0.19*	NS	NS	NS	NS
Energy intake	NS	NS	NS	NS	NS	NS
Calcium	NS	NS	NS	NS	NS	NS
Carbohydrates	NS	NS	NS	NS	NS	NS
Fat	NS	NS	NS	NS	NS	NS
Energy availability	NS	NS	NS	NS	NS	NS
IGF-1	NS	NS	NS	NS	NS	NS
Chronological age*Group	0.24 ± 0.09*	NS	NS	NS	NS	NS
Weight*Group	NS	NS	-	-	-	-
Height*Group	-	NS	-	-	-	-
Energy intake*Group	-	-	-	-	-	-
Calcium*Group	-	-	-	-	-	-
Carbohydrates*Group	-	-	-	-	-	-
Fat*Group	-	-	-	-	-	-
IGF-1*Group	-	-	-	-	-	-

Values are estimates ± standard errors
* p<0.05; ** p<0.01; *** p<0.001; significant predictor
Energy availability prediction was estimated considering only data from vocational dance students
NS: not significant
BMC: bone mineral content; BMD: bone mineral density; FN: femoral neck; LS: lumbar spine; FA: forearm; GH: growth hormone; IGF-1: insulin-like growth factor 1

DISCUSSION

It is widely believed that vocational dance students and professional dancers might be at risk of developing low BMD and osteoporosis later in life. Several studies have been giving support to this claim [44–46,146]. Additionally, the IADMS published a report highlighting dancers' bone health as a topic of major concern [17]. Nevertheless, apart from all these premises, less is known in relation to vocational dancer's bone heath (both in female and male young dancers) [109]. To my knowledge, the present study is the first to longitudinally assess female and male vocational dance students' during adolescence, and to investigate the association between bone mass accruals with nutrition and energy availability. It was found longitudinal evidence that young vocational dancers' BMC and BMD (both female and male) are lower compared to controls throughout time, and that nutrition (i.e. calcium, fat, carbohydrates) were not significant predictors of bone mass gains as dancers' growth; energy intake was found to positively predict bone mass accruals at the FN in female dancers.

Body characteristics are important parameters to be considered in a vocational dance school [13]. Therefore, it was not surprising to find that body weight was significantly lower in all measured occasions compared to controls in both female and male dancers. Actually, probably because professional dancing emphasises leanness and an aesthetic silhouette, it is general accepted that these professionals are at increased risk for a relative low energy availability (due to restricted diets to achieve the ideal body shape) [17–19], placing them at risk for the female athlete triad/ RED-S and, consequently, low BMD. However, as dancers' energy availability lied within the normal range throughout all measured occasions, it seems unlikely that this parameter will trigger the female athlete triad and/or RED-S. Indeed, energy deficiency and low energy intake have been linked to low BMD phenotypes through the hormone IGF-1; serum alterations of this hormone have been reported in the presence of negative energy imbalance [140]. As showed in the previous study (Study 3), IGF-1 serum concentrations of female vocational dancers were similar and even higher (at 2-yr follow-up) compared to controls. In male vocational dance students, the present study also showed that IGF-1 serum concentrations did not differ between dancers and matched controls. These facts reinforce the unlikelihood of our dancers to develop RED-S. Actually, if energy intake and/or energy availability were negatively affecting BMD in our vocational dancers, it would be expected to find

lower IGF-1 serum concentrations compared to controls as it has been reported that IGF-1 is sensitive to changes in nutrition intakes, particularly caloric intake [140]. In accordance, the aetiology of the lower bone health displayed by vocational dance students compared to controls might be triggered by other factors. Future studies should consider parameters such as genetics; further, dance participants and controls should be assign into normal BMD and low BMD to investigate whether energy intake and energy availability predict low bone mass phenotypes.

Studies in elite dance usually focus their analysis in female dancers; only a few studies included male dancers in their methodologies [109]. These studies focused on professional male dancers and show conflicting results, i.e. one showed that BMD in male dancers is lower compared with controls, whereas other showed higher BMD [64,69]. In study 2, it is suggested that young male dance students may also be at risk for low BMD as it was found that adjusted BMD values for body weight and maturation were significantly lower compared to controls at both impact and non-impact sites. In the present study male vocational dance students were followed longitudinally. Similar as their female counterparts, it was found an absence of a "catch-up" accrual during the follow-up in relation to controls; male dancers´ bone mass values remained consistently lower compared to non-exercising controls as they progressed on their professional dance training. Apparently, as in female dancers, traditional osteoporosis risk factors as body weight, maturation, energy availability and nutrition intake seem not to explain the lower bone mass values revealed by male dancers compared to controls. Actually, it is clear from the BPAQ that the amount of exercise relevant to bone was significant higher in both female and male dancers compared to controls. This means that our vocational dancers have been receiving more weight-bearing stimuli than controls. Consequently, due to the positive effects of this type of exercise on bone cells, it would be expected to find significantly higher bone mass accruals compared to controls [132]. Furthermore, it would also be expected to find significantly higher IGF-1 serum concentrations in vocational dancers compared to controls due to the effects of exercise in this hormone [140,150], leading further to greater bone mass accruals compared to non-exercise participants. Whereas at 2-yr follow-up, IGF-1 serum concentrations were significantly increased in our female vocational dancers compared to controls, the same was not observed in vocational male dancers. As previous hypothesised in

other studies in this Thesis, it could be the case that genetic mechanisms are mediating the degree of bone mass gains from exercise stimuli [153–155].

The present study has limitations. Results of the present paper are based on a mixed- longitudinal design with a relative small sample size for this type of study. The main outcome in this study – BMC, measured by DXA - does not account for changes in bone microarchitecture; however, this device (as well as BMC as main outcome) is considered the “gold standard” to longitudinally measure children [158]. The use of the use of a self-reported questionnaire to assess bone specific loading and nutrition intake, as well as the lack of data on energy availability in controls, are further limitations of the present study.

CONCLUSION

Female and male vocational dancers displayed lower bone mass values compared to controls at both impact and non-impact sites; dancers´ bone mass values remained consistently lower compared to non-exercising controls as they progressed on their professional dance training. Energy intake and energy availability was found to positively predict bone mass gains at the FN in female dancers. Nutrition, energy expenditure and energy availability seem not to explain the lower bone mass values revealed by male dancers compared to controls at both impact and non-impact sites.

CHAPTER 9: GENETIC VARIATION IN WNT/β-CATENIN AND ER SIGNALLING PATHWAYS IN PROFESSIONAL BALLET DANCERS AND VOCATIONAL DANCE STUDENTS: ASSOCIATIONS WITH LOW BONE MINERAL DENSITY (STUDY 5)

Parts of this chapter have been submitted in the peer-reviewed journal *Journal of Clinical Endocrinology and Metabolism*. The author of this Thesis appears as the leading author.

ABSTRACT

Background and Aim Genetic variation at the Wnt/β-catenin and ER signalling pathways are critical in determining bone mass phenotypes. As the risk factors associated with low BMD in dancers are not fully described, this study aims to assess if SNPs in the Wnt/β-catenin and ER signalling pathways are associated with low BMD in professional ballet dancers and vocational dance students.

Methods A genetic population-based association study was conducted in a cohort of professional and vocational dance students (n=151); age and sex-matched controls (non-dancers) were included. Eleven SNPs of the Wnt/β-catenin and ER pathways (*SOST*: rs851054, rs851056, rs10534024, rs4792909, rs9902563; *LRP5*: rs3736228, rs2306862, rs682429, rs491347, rs3781590, rs2508836, rs643892, rs312786; *ESR1*: rs2234693, rs9340799; *ESR2*: rs1256030, rs960070) were genotyped and evaluated for association with low BMD (measured by DXA and defined as *Z*-score < -1.0 for adults; and *Z*-score < -2.0 for adolescents) at the forearm, lumbar spine (LS) and femoral neck (FN).

Results Comparing dancers with normal BMD with dancers with low BMD, it was found the A allele of rs9340799 (*ERS1*) was associated with low BMD in dancers at the forearm [OR (CI)=10.74 (1.37-83.98), p=1.9x10^{-3}]. Considering controls with normal BMD and dancers with low BMD, the A allele of the same SNP increased significantly the odds of low BMD in dancers at the forearm [OR (CI)=1.95 (1.09-3.51), p=0.020], LS [OR (CI)=2.32 (1.24-4.32), p=5.8x10^{-3}] and FN [OR (CI)=2.45 (1.26-4.74), p=5.2x10^{-3}]. *LRP5* rs2508836 C allele was also associated with increased odds of low BMD in dancers at the LS (OR=6.90; CI, 1.27-37.49, p=0.010). Haplotype analysis revealed the blocks GCGT and GCAG at the *LRP5* gene significantly increased the odds for low BMD in dancers at the LS and forearm [OR (CI)=12.7 (1.22-132.18) p=0.033; OR (CI)= 6.43 (1.33-31.14, p=0.021], respectively.

Conclusion This study shows, for the first time, that genetic variants at the Wnt/β-catenin and ER signalling pathways are associated with increased odds for low BMD professional ballet dancers and vocational dance students.

Keywords BMD; athletes; sclerostin; weight-bearing; dance

INTRODUCTION

Osteoporosis is a bone disease caused by bone resorption exceeding bone formation [138]. The result of this bone remodelling imbalance is reduced bone mass and strength, changes in the microarchitecture of bone tissue and increased fracture risk [138]. BMD is the parameter most used to diagnose osteoporosis and predict bone fracture risk [32,33].

Osteoporosis is traditionally associated with the elderly and postmenopausal women [164]. In other populations, such as elite dancers, this clinical condition, along with low BMD, has been recognized as an increasingly important health concern [17]. Indeed, some observations have emphasized that professional and vocational dance students have low bone mass values compared to controls or normative values at several skeletal sites [18,25,43,146]. Literature highlights the possible deleterious effects of the risk factors for low bone mass that dancers are exposed to (e.g. low body weight, menstrual disturbances, low energy availability). Nevertheless, in previous studies of the present PhD thesis it has been shown that the aforementioned factors not always seem to be associated with BMD and low bone mass phenotypes. Indeed, it seems that the mechanisms and risk factors associated with low BMD in dancers are not fully understood [109].

Research on animal models and human populations has been showing that there is a high variation in the skeletal adaptation to exercise [153,154,165]. Such differential bone anabolic responses might rely on the genetic background [154,155,165]. Indeed, two biological signalling transduction pathways, the oestrogen receptor (ER) and the Wnt/β-catenin signalling pathway have an important role in mediating bone responsiveness to mechanical loading [132,155]. Therefore, SNPs in genes of the aforementioned pathways (e.g. *SOST*, *LRP5*, *ESR1* and *ESR2*) may be relevant and contribute to a further understanding of the factors involved in low bone mass phenotypes in dancers; however, this question has never been addressed. In accordance, the aim of the present study is to assess the association of genetic

polymorphisms in the Wnt/β-catenin and ER signalling pathways with low BMD in professional ballet dancers and vocational dance students.

METHODS

Study population

The present genetic population-based association study has been conducted in the community of active dancers from a professional ballet company and vocational dance students enrolled in a vocational dance school (school offering full-time professional dance training). An introductory letter describing briefly the study was sent to the executive boards of the dance school and ballet company. Following boards' permission, vocational dancers (and respective guardians) and professional dancers were presented with the purposes of the study; 126 dance students (70.0%) and 41 professional dancers (68.3%) volunteered. All volunteers completed a questionnaire concerning their ethnicity, medical history, and past/current calcium/vitamin D supplementation. Eligible criteria included participants of white European origin, with no illnesses or treatments that might affect bone metabolism, not taking medication known to influence bone metabolism and no calcium/vitamin D supplementation (two dance students and one professional dancers were excluded). Women taking oral contraceptives and hormonal therapy were excluded (one professional dancer). Based on these criteria, the studied population consisted of 151 professional ballet dancers and vocational dance students.

Non-exercising participants were recruited from two local state schools and local Universities to act as controls. Eligibility criteria for controls were set according to dancers' characteristics, i.e. controls were only considered eligible if they were of the same sex, age (defined as decimal age; 12-months difference of a dancer) and race (white European-Caucasian) as dancers. Exclusion criteria included participation in organised physical activities/sports outside school curriculum; children participants involved in physical education sessions at their school were not excluded (2 sessions of physical education lessons at school, 1/1.5 hours per session). Control participation was also restricted to those who had received/were receiving medications known to affect bone metabolism and to who reported illnesses/treatments that might affect bone metabolism. Following consent from the respective boards of directors, the study was advertised by the school/ universities

authorities. Out of the 282 responses (105 pupils, 177 university students), 151 that fulfilled the aforementioned criteria and were further included in the study.

All participants provided signed informed consent according to the Declaration of Helsinki. The study was approved by the ethics committee of the Regional Administration of Health of Lisbon, Portugal (Proc.063/CES/INV/2012).

Study design

Study design and data collection are summarised in Figure 8. Briefly, a cross-sectional study was conducted in all professional dancers and vocational dance students recruited for the present study (151 dancers); these dancers were screened for the presence of low BMD and traditional osteoporosis risk factors (i.e. low body weight, menstrual disturbances, and low energy availability) considering current guidelines on the topic. Furthermore, in order to investigate potential novel factors associated with low BMD in our dance population, a genetic association study was also conducted in all recruited professional dancers, vocational dance students and aged- and sex-matched controls (151 dancers and 151 controls). Afterwards, in order to obtain more evidence concerning previous findings, a subgroup of 101 vocational dance students (and aged- and sex-matched controls) were assessed longitudinally aiming at evaluating the associations of sclerostin with bone mass gains.

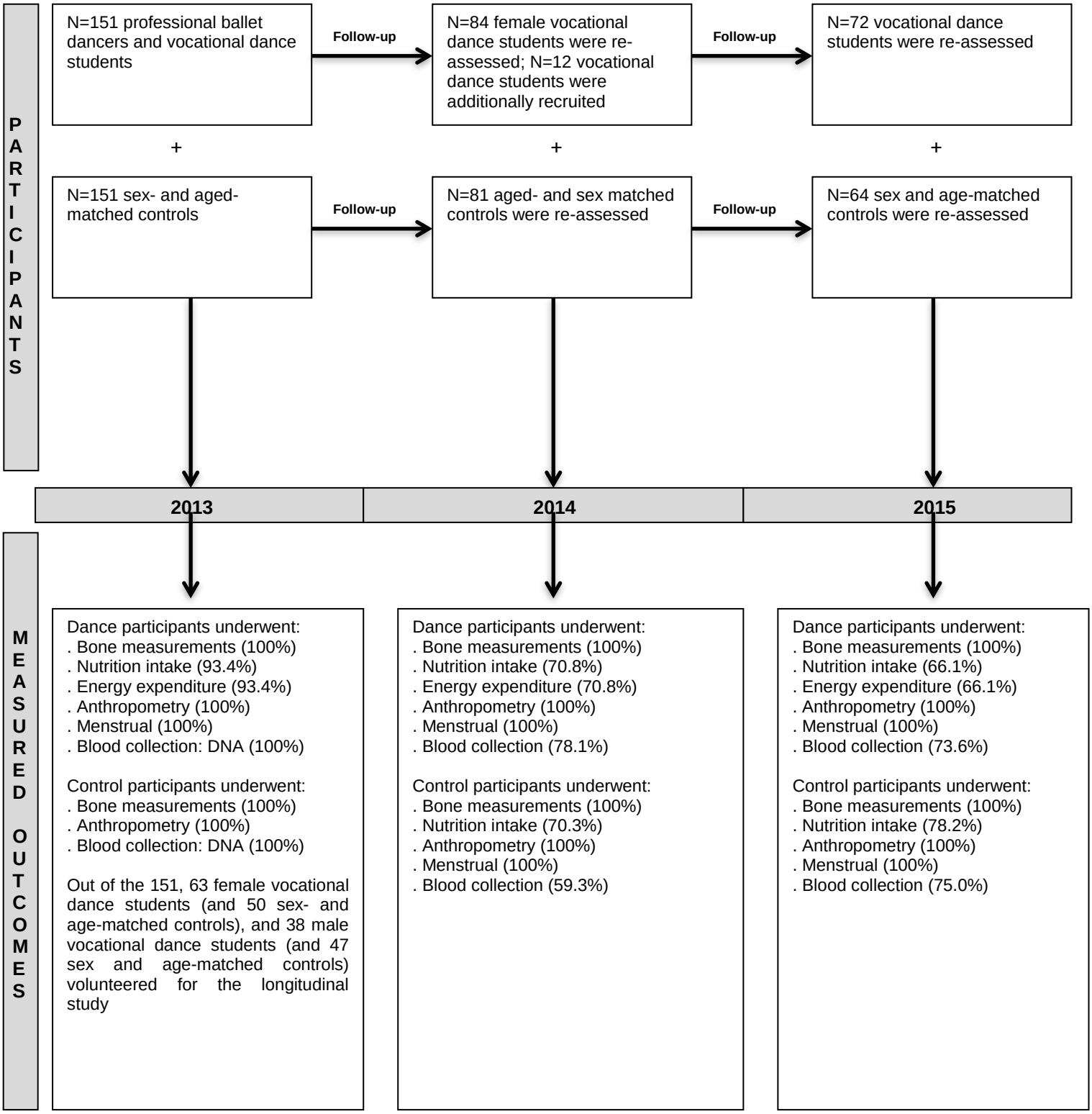


Figure 8. Flow chart of data collection and timeline

Cross-sectional analysis

All 151 professional ballet dancers and vocational dance students were screened for the presence of low BMD. The ISCD criterion for children was used to diagnose vocational dance students (the ISCD has adopted the term “low BMD” for a *Z*-score less than -2.0), and the ACSM guidelines were adopted to diagnose professional ballet dancers. The ACSM uses the term “low BMD” for a *Z*-score between -1.0 and -2.0 (along with secondary risk factors for stress fractures) and the term “osteoporotic” for a *Z*-score equal or less than -2.0 (along with secondary risk factors for stress fractures). In accordance, it was also searched for the presence of the aforementioned osteoporosis risk factors in our dance population. Specifically, dancers were screened for the presence of low body weight (defined as a body mass index of <18.5 for adult participants – WHO criteria; for children and adolescents, it was considered the body mass index expected for the age), low energy availability (<30.0 kcal/kgFFM/day), and, in case of female participants, menstrual disturbances (primary/secondary amenorrhea, oligoamenorrhea).

Genetic association study

Genes of the Wnt/β-catenin and ER signalling pathways with potential biological function, and further established in genome-wide association studies (GWAS) and meta-analysis as genes related to low bone mass phenotypes, were identified according to literature reports. This resulted in the identification of four major genes: *SOST*, *LRP5* (Wnt/β-catenin pathway), *ESR1* and *ESR2* (ER signalling pathway). SNPs in or near these genes reported to have a significant association with BMD variation and risk of osteoporosis in European populations were identified using genetic variation databases such as Hapmap and NCBI, and previous association studies on European populations. The following SNPs were identified in *SOST*: rs851054, rs851056, rs10534024, rs4792909, rs9902563; *LRP5*: rs3736228, rs2306862, rs682429, rs491347, rs3781590, rs2508836, rs643892, rs312786; *ESR1*: rs2234693, rs9340799; *ESR2*: rs1256030, rs960070.

Characteristics of each SNP were examined using the Ensembl database, and linkage disequilibrium (LD) analyses were performed using Haploview 4.1 with data available on HapMap. Within *SOST* region, rs4792909 and rs9902563 were located 32275 and 74118 bases, respectively, far downstream of the *SOST* start site. The SNPs rs851054 and rs851056 were in complete LD (R^2=1), while rs10534025 were

not in LD with any of the other SNPs. Both rs3781590 and rs643892 in *LRP5* were in complete LD (R^2=1) with another SNP that has not been previously studied in relation to bone mass phenotypes (rs587808), whereas rs2508836, rs491347 and rs312786 were not in LD with any of the others SNPs. Information on rs682429 was not available on HapMap by the time of the LD assessment. The minor allele frequency (MAF) of rs3736228 and rs2306862 was low within the European ancestry population (CEU) (0.12 and 0.13, respectively). Considering the SNPs in *ESR1*, LD analyses revealed that rs2234693 and rs9340799 were in moderate LD (R^2=0.6), whereas rs1256030 and rs960070 in *ESR2* revealed high LD (R^2=0.9). Based on the attributes described, the following eleven SNPs were selected for genotyping: *SOST*: rs851054, rs10534024; *LRP5*: rs682429, rs491347, rs2508836, rs587808, rs312786; *ESR1*: rs2234693, rs9340799; *ESR2*: rs1256030, rs960070.

Longitudinal analysis

All vocational dance students (n=115) and aged- and sex matched controls included in the cross-sectional analysis were asked to participate on a follow-up study in order to analyse sclerostin serum concentration and bone mass throughout growth. Sixty-three female vocational dancers (vs. 50 aged- and sex-matched controls) and 38 male vocational dance students (vs. 47 aged- and sex-matched controls) volunteered. A mixed-longitudinal design was followed. Data were collected annually for three consecutive years, from January 2013 to March 2015. Details on the participants' measurements and specific methodology appear in Figure 9.

Anthropometry, menstrual, nutritional intake and energy availability

The same procedures as the previous study (Study 8) were followed.

Hormonal analysis

Blood samples were collected in early morning after an 8-hour fasting. Blood samples were submitted to centrifugation at 2500g for 10 min; serum samples were stored at -80ºC until they were analysed. Serum sclerostin concentrations were measured by an ELISA assay kit (Human SOST/Sclerostin Quantikine ELISA Kit, Ref DSST00), from R&D Systems, Inc. (Minneaplolis, MN 55413, USA). The intra-assay and inter-assay CV's ranged between 1.8-2.1% and 8.2-10.8%, respectively.

Genotyping

Genomic DNA was isolated from blood using the MagNA Pure LC DNA isolation kit (Roche, Switzerland) according to product specifications. Primers were generated from the genomic sequence using Primer-BLAST and its specificity determined using BLASTn. DNA was amplified with the QIAGEN Multiplex PCR Kit (Qiagen, Germany), either in single PCR reactions (SNP rs312786) or in two sets of multiplex reactions (set 1: SNPs rs2234693, rs960070, rs682429, rs587808 and rs851054; set 2: SNPs rs9340799, rs1256030, rs491347, rs2508836 and rs10534024). PCR products were purified using Sephadex G-50 fine (Sigma-Aldrich, USA) columns on a filtration plate and genotypes determined using the Genetic Analyzer 3130 and 3130xl (Applied Biosystems).

Bone measurements

The same procedures as the previous studies (Study 7 and 8) were followed.

Statistical analyses

Cross-sectional analysis: Independent t-tests were used to compare descriptive characteristics and unadjusted values of bone measurements between dancers with low BMD and dancers with normal BMD. Bone parameters were further compared between professional dancers with low BMD with professionals with normal BMD after adjustment for age, sex and primary amenorrhea using analysis of covariance (ANCOVA). ANCOVA was also used to estimate bone mass values in pre-professional dance students' bone mass values after the adjustment for sex, energy availability, fat, calcium and carbohydrates intakes. All analyses were performed with SPSS v.20.0 and statistical significance was set at $p<0.05$.

Genetic association study: Independent t-tests were used to compare general characteristics between dancers and controls (stratified by bone mass phenotypes) using the software SPSS (version 18.0). Hardy-Weinberg equilibrium (HWE) of alleles at individual loci (level of significance set at $p<0.01$) was measured at the level of the control population. Association of genotypes with study groups (defined according to bone mass status: dancers with normal BMD vs. dancers with low BMD, and controls with normal BMD vs. dancers with low BMD) and independence of SNPs were assessed by unconditional logistic regression with the "SNPassoc" package implemented in R. The minor allele of most SNPs is the ancestral allele and,

thus, it has been selected as the reference allele in all analysis. Four hereditary models were considered in the analysis (codominant, dominant, recessive and log-additive) and included the variable weight. Other confounding variables such as sex and age were not included in the models because the BMD measurements were performed according to references that already included adjustment for those variables. The adjustment for multiple testing was performed by the false discovery rate (FDR) method. Haplotype frequencies were inferred using the “haplo.stats” package implemented in R. Haplotype association with the study groups (OR, 95% CI and *p* values) was assessed for those with a minimum haplotype frequency of 0.01 and using as reference the most frequent haplotype.

Longitudinal analysis: Independent t-tests were used to compare general characteristics between vocational dance students and aged- and sex-matched controls at each measured occasion. Bone mass values were adjusted for sex and serum sclerostin concentrations using ANCOVA. Significance was set at $p<0.05$.

RESULTS

Out of the 151 professional ballet dancers and vocational dance students included in the present study, 80 were diagnosed with low BMD (against 70 that were within the normal range) (Figure 10). Out of these 80, 56.3% had at least one traditional osteoporosis risk factor, whereas 28.6% were diagnosed with low BMD but did not display any risk factor. In contrast, 37.2% revealed one or more traditional osteoporosis risk factors, but had normal BMD (Figure 9).

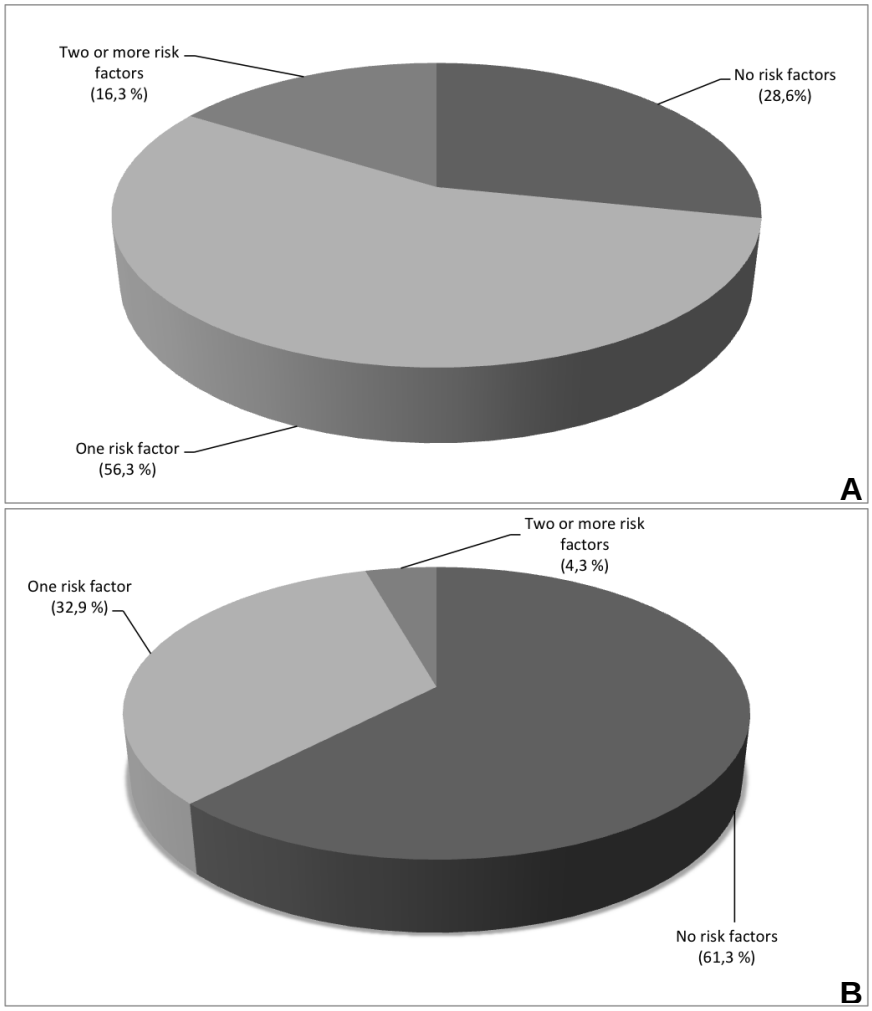


Figure 9. (A) Professional ballet dancers and vocational dance students with low bone mineral density (BMD) at least in one anatomical site and absence/ presence of osteoporosis risk factors (N=80). (B) Professional ballet dancers and vocational dance students with normal BMD and absence/ presence of osteoporosis risk factors frequently considered in athletic populations (N=70).
The following osteoporosis risk factors were considered: low body weight (defined as a body mass index of <18.5 for adult participants – WHO criteria; for children and adolescents, it was considered the body mass index expected for the age), low energy availability (<30.0 kcal/kgFFM/day), and, in case of female participants, menstrual disturbances (primary/secondary amenorrhea, oligoamenorrhea).

General characteristics of these participants are displayed in Table 14 (Appendix 7). Briefly, considering professional dancers, the ones with low BMD had approximately 37 years old, whereas the ones with normal BMD had approximately 30 years old (p<0.05). The percentage of female professional dancers displaying primary amenorrhea was significantly higher in the ones with normal BMD compared with the ones with low BMD (p<0.05). There was not a significant difference between professional dancers with low versus normal BMD in terms of energy, calcium, fat and carbohydrates intakes, nor in energy availability. BMC and BMD were significantly lower at FN and forearm in professional dancers with low BMD

compared with the ones with normal BMD values (p<0.01). Regarding vocational dance students, there was not a significant difference in age, weight and height between the ones with low BMD with the ones with normal BMD. However, vocational dancers with low BMD consumed significantly less calories (p<0.01), calcium (p<0.01), fat (p<0.05) and carbohydrates (p<0.01) compared with vocational dancers with normal BMD. Also, although within the normal range, energy availability of vocational dancers with low BMD is significantly lower compared with the ones with normal BMD (p<0.05). All bone mass parameters were significantly lowered in vocational dancers with low BMD compared to the ones with normal BMD (BMC values: p<0.001; BMD values: p<0.01).

Bone mass parameters of professional dancers were further adjusted for age, sex and primary amenorrhea, whereas in vocational dancers bone values were adjusted for sex, energy availability and nutrition intake (energy, fat, calcium, carbohydrates) (Table 15; Appendix 7). After the adjustment, professional dancers previously diagnosed with low BMD continued to display a significant lower BMC and BMD values at the forearm compared with their counterparts with normal BMD (BMC: p<0.05; BMD: p<0.01). Similarly, vocational dancers with low BMD also displayed significantly lower bone mass parameters compared with vocational dancers with normal BMD at all anatomical sites after the adjustment (FN BMC/ BMD: p<0.001; LS BMC:p<0.001;forearmBMD:p<0.001).

Characteristics of the participants included in the genetic analysis appear in Table 16 (Appendix 7). Three SNPs [rs682429 (*LRP5*), rs851054 and rs10534024 (*SOST*)] that were significantly deviated from the Hardy-Weinberg Equilibrium (p<0.01) were not considered for statistical analysis and will not be further mentioned (Table 17; Appendix 7). Table 18 shows the association of SNPs in *LRP5*, *ESR1* and *ESR2* with bone mass phenotypes comparing professional and vocational dancers with normal BMD (reference group – dancers with normal BMD) and dancers with low BMD according to different hereditary models. The T allele in rs2234693 (*ESR1*) was associated with low BMD at the LS [OR (CI)=1.77 (1.06-2.97), p=0.026). However, this association was not retained after FDR correction. Considering the SNP rs9340799 (*ESR1*), the A allele (dominant model) was associated with low BMD in professional and vocational at the forearm [OR (CI)=10.74 (1.37-83.98), p=1.9x10^{-3}]. The genotypes AG and AA were significantly associated with low BMD at the forearm with an OR (CI) of 10.63 and 10.85, respectively (p=8.2x10^{-3}).

Table 18. Genotype distribution of SNPs in the Wnt/**β**-catenin and ER signalling pathways and association with low BMD phenotype in dancers [dancers with normal BMD (ref) versus dancers with low BMD] given by the odds ratio (OR) and 95% confidence intervals (95%CI).

Gene/ SNP	Model	Dancers						OR (95% CI) – *p*-value					
		Normal BMD			Low BMD								
		Forearm	LS	FN	Forearm	LS	FN	Forearm	*p*	LS	*p*	FN	*p*
LRP5		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)						
rs491347	Codominant												
	GG	13(13.3)	11(11.5)	13(13.1)	6(12.0)	8(15.1)	6(12.5)	1.00 *	0.896	1.00 *	0.877	1.00 *	0.978
	AG	32(32.7)	34(35.4)	35(35.4)	19(38.0)	18(34.0)	17(35.4)	1.16(0.37-3.64)		0.80(0.27-2.37)		1.13(0.36-3.53)	
	AA	53(54.1)	51(53.1)	51(51.5)	25(50.0)	27(50.9)	25(52.1)	0.98(0.33-2.90)		0.76(0.27-2.14)		1.10(0.37-3.26)	
	A-carrier vs GG	85(86.7)	85(88.5)	86(86.9)	44(88.0)	45(84.9)	42(87.5)	1.05(0.37-2.98)	0.932	0.78(0.29-2.09)	0.618	1.11(0.39-3.16)	0.842
	AA vs G-carrier	53(54.1)	51(53.1)	51(51.5)	25(50.0)	27(50.9)	25(52.1)	0.87(0.44-1.74)	0.696	0.90(0.46-1.76)	0.753	1.01(0.50-2.01)	0.984
	Log-additive	98(66.2)	96(64.4)	99(67.3)	50(33.8)	53(35.6)	48(32.7)	0.94(0.58-1.54)	0.813	0.89(0.56-1.44)	0.646	1.03(0.63-1.68)	0.914
rs2508836	Codominant												
	TT	12(12.2)	12(12.5)	12(12.1)	2(4.0)	2(3.8)	2(4.2)	1.00 *	0.194	1.00 *	0.169	1.00 *	0.154
	CT	37(37.8)	36(37.5)	35(35.4)	22(44.0)	23(43.3)	23(47.9)	3.76(0.76-18.65)		3.83(0.78-18.87)		3.91(0.80-19.18)	
	CC	49(50.0)	48(50.0)	52(52.5)	26(52.0)	28(52.8)	23(47.9)	3.22(0.66-15.64)		3.60(0.74-17.46)		2.69(0.55-13.05)	
	C-carrier vs TT	86(87.8)	84(87.5)	87(87.9)	48(96.0)	51(96.2)	46(95.8)	3.45(0.73-16.21)	0.078	3.70(0.79-17.38)	0.060	3.19(0.68-14.92)	0.099
	CC vs T-carrier	49(50.0)	48(50.0)	52(52.5)	26(52.0)	28(52.8)	23(47.9)	1.05(0.53-2.09)	0.890	1.15(0.59-2.27)	0.682	0.85(0.42-1.70)	0.641
	Log-additive	98(66.2)	96(64.4)	99(67.3)	50(33.8)	53(35.6)	48(32.7)	1.26(0.74-2.14)	0.399	1.34(0.79-2.29)	0.270	1.09(0.64-1.86)	0.738
rs587808	Codominant												
	GG	16(16.3)	19(19.8)	19(19.2)	12(24.0)	9(17.0)	9(18.8)	1.00 *	0.497	1.00 *	0.923	1.00 *	0.957
	AG	40(40.8)	38(39.6)	40(40.4)	20(40.0)	23(43.4)	21(43.8)	0.71(0.28-1.80)		1.21(0.47-3.16)		1.07(0.41-2.79)	
	AA	42(42.9)	39(40.6)	40(40.4)	18(36.0)	21(39.6)	18(37.5)	0.57(0.22-1.45)		1.14(0.44-3.00)		0.95(0.36-2.51)	
	A-carrier vs GG	82(83.7)	77(80.2)	80(80.8)	38(76.0)	44(83.0)	39(81.2)	0.63(0.27-1.48)	0.297	1.18(0.49-2.85)	0.712	1.01(0.42-2.45)	0.984
	AA vs G-carrier	42(42.9)	39(40.6)	40(40.4)	18(36.0)	21(39.6)	18(37.5)	0.71(0.35-1.45)	0.350	1.00(0.50-2.00)	0.999	0.91(0.45-1.85)	0.793
	Log-additive	98(66.2)	96(64.4)	99(67.3)	50(33.8)	53(35.6)	48(32.7)	0.76(0.48-1.21)	0.242	1.05(0.66-1.66)	0.845	0.96(0.60-1.54)	0.871
rs312786	Codominant												
	TT	8(8.2)	10(10.4)	10(10.1)	5(10.0)	3(5.7)	3(6.2)	1.00 *	0.406	1.00 *		1.00 *	0.126
	GT	36(36.7)	36(37.5)	34(34.3)	23(46.0)	23(43.4)	25(52.1)	1.07(0.31-3.69)		2.06(0.51-8.36)	0.568	2.40(0.60-9.66)	
	GG	54(55.1)	50(52.1)	55(55.6)	22(44.0)	27(50.9)	20(41.7)	0.66(0.19-2.25)		1.77(0.45-7.02)		1.19(0.30-4.80)	
	G-carrier vs TT	90(91.8)	86(89.6)	89(89.9)	45(90.0)	50(94.3)	45(93.8)	0.82(0.25-2.67)	0.180	1.89(0.49-7.25)	0.331	1.65(0.43-6.33)	0.446
	GG vs T-carrier	54(55.1)	50(52.1)	55(55.6)	22(44.0)	27(50.9)	20(41.7)	0.62(0.31-1.25)	0.242	0.96(0.49-1.89)	0.913	0.57(0.28-1.15)	0.116
	Log-additive	98(66.2)	96(64.4)	99(67.3)	50(33.8)	53(35.6)	48(32.7)	0.73(0.43-1.24)	0.243	1.09(0.65-1.85)	0.744	0.79(0.46-1.34)	0.377
ESR1													
rs2234693	Codominant												
	CC	20(20.4)	22(22.9)	20(20.2)	8(16.0)	6(11.3)	7(14.6)	1.00 *	0.805	1.00 *	0.081	1.00 *	0.293
	TC	50(51.0)	51(53.1)	54(54.5)	28(56.0)	27(50.9)	23(47.9)	1.37(0.53-3.53)		2.04(0.73-5.68)		1.25(0.46-3.37)	
	TT	28(28.6)	23(24.0)	25(25.3)	14(28.0)	30(37.7)	18(37.5)	1.23(0.43-3.51)		3.30(1.11-9.86)		2.09(0.73-6.02)	
	T-carrier vs CC	78(79.6)	74(77.1)	79(79.8)	42(84.0)	47(88.7)	41(85.4)	1.32(0.53-3.27)	0.547	2.43(0.91-6.51)	0.062	1.52(0.59-3.90)	0.379
	TT vs C-carrier	28(28.6)	23(24.0)	25(25.3)	14(28.0)	20(37.7)	18(37.5)	0.97(0.45-2.09)	0.941	1.92(0.92-4.00)	0.081	1.77(0.84-3.73)	0.132
	Log-additive	98(66.2)	96(64.4)	99(67.3)	50(33.8)	53(35.6)	48(32.7)	1.08(0.65-1.79)	0.769	1.77(1.06-2.97)	0.026	1.49(0.88-2.50)	0.130
rs9340799	Codominant												
	GG	17(17.3)	14(14.6)	15(15.2)	1(2.0)	4(7.5)	3(6.2)	**1.00** *	**8.2x10^{-3}** **	1.00 *	0.278	1.00 *	0.067
	AG	41(41.8)	43(44.8)	46(46.5)	24(48.0)	22(41.5)	18(37.5)	**10.63(1.32-85.87)**		1.75(0.51-6.01)		1.92(0.49-7.48)	
	AA	40(40.8)	39(40.6)	38(38.4)	25(50.0)	27(50.9)	27(56.2)	**10.85(1.35-87.36)**		2.47(0.73-8.41)		3.60(0.94-13.73)	
	A-carrier vs GG	81(82.7)	82(85.4)	84(84.8)	49(98.0)	49(92.5)	45(93.8)	**10.74(1.37-83.98)**	**1.9x10^{-3}** **	2.09(0.65-6.75)	0.196	2.67(0.73-9.74)	0.106
	AA vs G-carrier	40(40.8)	39(40.6)	38(38.4)	25(50.0)	27(50.9)	27(56.2)	1.41(0.71-2.81)	0.330	1.58(0.80-3.12)	0.191	2.12(1.05-4.29)	0.035
	Log-additive	98(66.2)	43(44.8)	99(67.3)	50(33.8)	22(41.5)	48(32.7)	1.74(1.01-2.97)	0.039	1.51(0.90-2.54)	0.115	1.89(1.08-3.28)	0.020

Table 18. Continued

Gene/ SNP	Model	Dancers						OR (CI) – *p*-Value					
		Normal BMD			Low BMD								
		Forearm	LS	FN	Forearm	LS	FN	Forearm	*p*	LS	*p*	FN	*p*
ESR2		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)						
rs1256030	Codominant												
	AA	19(19.4)	19(19.8)	18(18.2)	11(22.0)	11(20.8)	11(22.9)	1.00 *	0.250	1.00 *	0.833	1.00 *	0.420
	GA	50(51.0)	47(49.0)	51(51.5)	19(38.0)	23(43.4)	19(39.6)	0.65(0.26-1.62)		0.85(0.35-2.10)		0.61(0.24-1.54)	
	GG	29(29.6)	30(31.2)	30(30.3)	20(40.0)	19(35.8)	18(37.5)	1.24(0.48-3.20)		1.07(0.42-2.76)		0.97(0.37-2.52)	
	G-carrier vs AA	79(80.6)	77(80.2)	81(81.8)	39(78.0)	42(79.2)	37(77.1)	0.86(0.37-2.00)	0.725	0.94(0.41-2.17)	0.880	0.75(0.32-1.75)	0.504
	GG vs A-carrier	29(29.6)	30(31.2)	30(30.3)	20(40.0)	19(35.8)	18(37.5)	1.67(0.81-3.44)	0.166	1.20(0.59-2.44)	0.622	1.36(0.66-2.81)	0.413
	Log-additive	98(66.2)	47(49.0)	99(67.3)	50(33.8)	53(35.6)	48(32.7)	1.19(0.74-1.93)	0.474	0.82(0.41-1.61)	0.810	0.63(0.31-1.26)	0.868
rs960070	Codominant												
	GG	23(23.5)	25(26.0)	24(24.2)	12(24.0)	10(18.9)	10(20.8)	1.00 *		1.00 *	0.671	1.00 *	0.427
	CG	52(53.1)	48(50.0)	54(54.5)	24(48.0)	29(54.7)	23(47.9)	0.94(0.40-2.23)	0.809	1.45(0.60-3.46)		0.99(0.41-2.41)	
	CC	23(23.5)	23(24.0)	21(21.2)	14(28.0)	14(26.4)	15(31.2)	1.24(0.47-3.28)		1.47(0.54-3.98)		1.68(0.62-4.53)	
	C-carrier vs GG	75(76.5)	71(74.0)	75(75.8)	38(76.0)	43(81.1)	38(79.2)	1.03(0.46-2.32)	0.940	1.45(0.63-3.34)	0.372	1.18(0.51-2.74)	0.695
	CC vs G-carrier	23(23.5)	23(24.0)	21(21.2)	14(28.0)	14(26.4)	15(31.2)	1.29(0.59-2.81)	0.525	1.13(0.52-2.46)	0.753	1.69(0.77-3.68)	0.192
	Log-additive	98(66.2)	96(64.4)	99(67.3)	50(33.8)	53(35.6)	48(32.7)	1.12(0.68-1.83)	0.657	1.20(0.74-1.96)	0.463	1.32(0.79-2.19)	0.287

* reference estimate

** statistical significance retained after FDR correction

The distribution of genotypes in controls with normal BMD (reference group – controls with normal BMD) and dancers with low BMD and the association with bone mass phenotypes are shown in Table 19. Considering SNP rs2508836 (*LRP5*), the C allele was associated with low BMD in dancers (dominant model) at the LS [OR (CI)=6.90 (1.27-37.49), p=1.0x10^{-3}]. Considering SNP rs9340799 (*ESR1)*, there is a significant difference in genotype frequencies between normal controls and dancers with low BMD at the forearm (p=0.019), LS (p=0.021) and FN (p=0.020). The A allele (log-additive model) significantly increased the odds of low BMD in professional and vocational dancers at the forearm [OR (CI)=1.95 (1.09-3.51), p=0.020], LS [OR (CI)=2.32 (1.24-4.32), p=5.8x10^{-3}] and FN [OR (CI)=2.45 (1.26-4.74), p=5.2x10^{-3}]. The association of the A allele with low BMD in dancers was also observed in the dominant model at the forearm [OR (CI)=8.37 (1.07-65.26), p=7.0x10^{-3}], and in the recessive model at the LS [OR (CI)=2.98 (1.30-6.87), p=9.3x10^{-3}] and FN [OR (CI)=3.08 (1.26-7.51), p=0.012]. All of the aforementioned associations regarding rs9340799 SNP were retained after FDR correction. Significant associations between genotypes and bone mass phenotypes regarding controls with normal BMD and controls with low BMD will only be reported here and not in tables. The only significant association found and retained after FDR correction was at the LS for SNP rs9340799 in *ESR1*. The A allele was significantly associated with low BMD [OR (CI)=2.10 (1.22-3.62), p=5.4x10^{-3}], an association already observed with dancers with low BMD.

Table 19. Genotype distribution of SNPs in the Wnt/β-catenin and ER signalling pathways and association with low BMD phenotype in dancers [controls with normal BMD (ref) versus dancers with low BMD] given by the odds ratio (OR) and 95% confidence intervals (95%CI).

Gene/ SNP	Model	Controls			Dancers			OR (CI) – P Value					
		Normal BMD			Low BMD								
		Forearm	LS	FN	Forearm	LS	FN	Forearm	*p*	LS	*p*	FN	*p*
LRP5		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)						
rs491347	Codominant												
	GG	12(9.8)	12(10.9)	9(9.1)	6(12.0)	8(15.1)	6(12.5)	1.00 *	0.919	1.00 *	0.608	1.00 *	0.675
	AG	43(35.2)	34(30.9)	32(32.2)	19(38.0)	18(34.0)	17(35.4)	0.89(0.27-2.96)		1.06(0.31-3.67)		0.67(0.16-2.76)	
	AA	67(54.9)	64(58.2)	58(58.6)	25(50.0)	27(50.9)	25(52.1)	0.80(0.25-2.54)		0.70(0.22-2.24)		0.55(0.15-2.09)	
	A-carrier vs GG	110(90.2)	98(89.1)	90(90.6)	44(88.0)	45(84.9)	42(87.5)	0.84(0.28-2.53)	0.754	0.82(0.27-2.48)	0.721	0.59(0.16-2.13)	0.426
	AA vs G-carrier	67(54.9)	64(58.2)	58(58.6)	25(50.0)	27(50.9)	25(52.1)	0.87(0.42-1.80)	0.714	0.67(0.30-1.48)	0.321	0.74(0.31-1.75)	0.491
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	53(35.6)	48(32.7)	0.90(0.53-1.51)	0.681	0.79(0.46-1.36)	0.395	0.77(0.42-1.40)	0.384
rs2508836	Codominant												
	TT	16(13.1)	18(16.4)	14(14.1)	2(4.0)	2(3.8)	2(4.2)	1.00 *	0.158	1.00 *	0.029	1.00 *	0.234
	CT	55(45.1)	46(41.8)	44(44.4)	22(44.0)	23(43.3)	23(47.9)	2.84(0.56-14.37)		5.99(1.05-34.26)		3.07(0.50-18.87)	
	CC	51(41.8)	46(41.8)	41(41.4)	26(52.0)	28(52.8)	23(47.9)	4.01(0.80-20.21)		7.89(1.39-44.97)		4.26(0.69-26.38)	
	C-carrier vs TT	106(86.9)	92(83.6)	85(85.9)	48(96.0)	51(96.2)	46(95.8)	3.38(0.70-16.31)	0.090	**6.90(1.27-37.49)**	**0.010** **	3.59(0.62-20.92)	0.122
	CC vs T-carrier	51(41.8)	46(41.8)	41(41.4)	26(52.0)	28(52.8)	23(47.9)	1.65(0.79-3.41)	0.179	1.76(0.80-3.90)	0.159	1.63(0.69-3.86)	0.267
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	53(35.6)	48(32.7)	1.68(0.94-3.02)	0.075	1.98(1.06-3.69)	0.027	1.70(0.85-3.40)	0.123
rs587808	Codominant												
	GG	24(19.7)	21(19.1)	18(18.2)	12(24.0)	9(17.0)	9(18.8)	1.00 *	0.690	1.00 *	0.615	1.00 *	0.920
	AG	49(40.2)	45(40.9)	45(45.5)	20(40.0)	23(43.4)	21(43.8)	0.94(0.36-2.43)		1.52(0.50-4.58)		1.05(0.32-3.41)	
	AA	49(40.2)	44(40.0)	36(36.4)	18(36.0)	21(39.6)	18(37.5)	0.70(0.27-1.81)		1.01(0.34-3.04)		0.86(0.26-2.88)	
	A-carrier vs GG	98(80.3)	89(80.9)	81(81.8)	38(76.0)	44(83.0)	39(81.2)	0.81(0.34-1.90)	0.629	1.23(0.45-3.36)	0.682	0.96(0.32-2.84)	0.936
	AA vs G-carrier	49(40.2)	44(40.0)	36(36.4)	18(36.0)	21(39.6)	18(37.5)	0.72(0.34-1.53)	0.394	0.77(0.34-1.71)	0.518	0.83(0.34-2.02)	0.688
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	53(35.6)	48(32.7)	0.83(0.52-1.32)	0.422	0.95(0.56-1.59)	0.835	0.91(0.51-1.64)	0.757
rs312786	Codominant												
	TT	10(8.2)	11(10.0)	9(9.1)	5(10.0)	3(5.7)	3(6.2)	1.00 *	0.474	1.00 *	0.959	1.00 *	0.799
	GT	56(45.9)	45(40.9)	46(46.5)	23(46.0)	23(43.4)	25(52.1)	0.45(0.12-1.65)		1.15(0.23-5.65)		0.58(0.11-3.21)	
	GG	56(45.9)	54(49.1)	44(44.4)	22(44.0)	27(50.9)	20(41.7)	0.58(0.16-2.09)		1.24(0.25-6.01)		0.70(0.13-3.92)	
	G-carrier vs TT	112(91.8)	99(90.0)	90(90.0)	45(90.0)	50(94.3)	45(93.8)	0.51(0.15-0.175)	0.294	1.19(0.26-5.54)	0.820	0.64(0.12-3.31)	0.597
	GG vs T-carrier	56(45.9)	54(49.1)	44(44.4)	22(44.0)	27(50.9)	20(41.7)	1.12(0.54-2.33)	0.758	1.10(0.50-2.40)	0.816	1.13(0.48-2.68)	0.781
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	53(35.6)	48(32.7)	0.94(0.53-1.67)	0.822	1.09(0.58-2.06)	0.778	1.00(0.49-2.04)	0.994
ESR1													
rs2234693	Codominant												
	CC	35(28.7)	35(31.8)	32(32.3)	8(16.0)	6(11.3)	7(14.6)	1.00 *	0.296	1.00 *	0.090	1.00 *	0.134
	TC	51(41.8)	45(40.9)	39(39.4)	28(56.0)	27(50.9)	23(47.9)	2.08(0.80-5.44)		2.49(0.81-7.65)		2.03(0.66-6.24)	
	TT	36(29.5)	30(27.3)	28(28.3)	14(28.0)	30(37.7)	18(37.5)	1.48(0.51-4.32)		3.63(1.09-12.09)		3.34(1.00-11.23)	
	T-carrier vs CC	87(71.3)	75(68.2)	67(67.7)	42(84.0)	47(88.7)	41(85.4)	1.84(0.74-4.59)	0.178	2.87(0.99-8.33)	0.041	2.47(0.87-7.01)	0.079
	TT vs C-carrier	36(29.5)	30(27.3)	28(28.3)	14(28.0)	20(37.7)	18(37.5)	0.89(0.40-2.00)	0.777	1.90(0.80-4.49)	0.145	2.12(0.82-5.46)	0.119
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	53(35.6)	48(32.7)	1.17(0.71-1.94)	0.544	1.83(1.03-3.26)	0.035	1.81(1.00-3.30)	0.046
rs9340799	Codominant												
	GG	25(20.5)	25(22.7)	22(22.2)	1(2.0)	4(7.5)	3(6.2)	**1.00** *	**0.019** **	**1.00** *	**0.021** **	**1.00** *	**0.020** **
	AG	54(44.3)	54(49.1)	44(44.4)	24(48.0)	22(41.5)	18(37.5)	**7.28(0.91-58.54)**		**1.88(0.51-6.98)**		**2.39(0.56-10.23)**	
	AA	43(35.2)	31(38.2)	33(33.3)	25(50.0)	27(50.9)	27(56.2)	**9.85(1.22-79.76)**		**4.86(1.27-18.56)**		**5.91(1.38-25-38)**	
	A-carrier vs GG	97(79.5)	85(77.3)	77(77.8)	49(98.0)	49(92.5)	45(93.8)	**8.37(1.07-65.26)**	**$7.0x10^{-3}$** **	2.84(0.82-9.85)	0.080	3.72(0.94-14.69)	0.041
	AA vs G-carrier	43(35.2)	31(28.2)	33(33.3)	25(50.0)	27(50.9)	27(56.2)	1.75(0.84-3.65)	0.136	**2.98(1.30-6.87)**	**$9.3x10^{-3}$** **	**3.08(1.26-7.51)**	**0.012** **
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	22(41.5)	48(32.7)	**1.95(1.09-3.51)**	**0.020** **	**2.32(1.24-4.32)**	**$5.8x10^{-3}$** **	**2.45(1.26-4.74)**	**$5.2x10^{-3}$** **

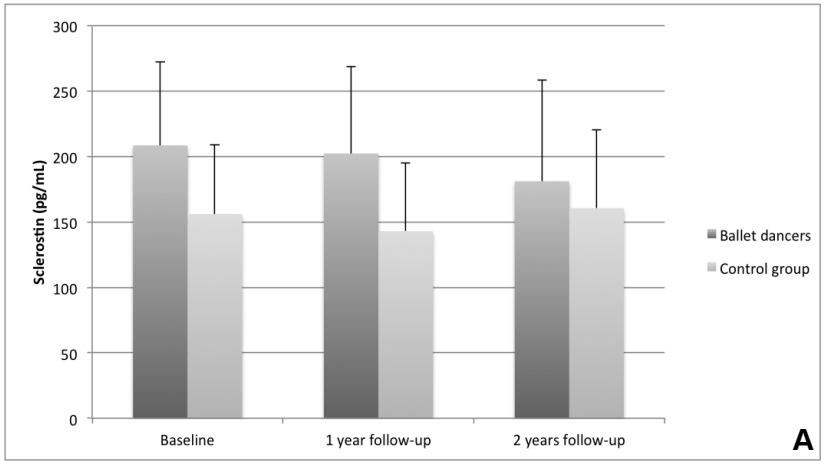
Table 19. Continued

Gene/ SNP	Model	Controls			Dancers			OR (CI) – P Value					
		Normal BMD			Low BMD								
		Forearm	LS	FN	Forearm	LS	FN	Forearm	*p*	LS	*p*	FN	*p*
ESR2		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)						
rs1256030	Codominant												
	AA	14(11.5)	11(10.0)	11(11.1)	11(22.0)	11(20.8)	11(22.9)	1.00 *	0.148	1.00 *	0.067	1.00 *	0.295
	GA	61(50.0)	52(47.3)	51(51.5)	19(38.0)	23(43.4)	19(39.6)	0.35(0.12-1.00)		0.33(0.10-1.11)		0.40(0.11-1.39)	
	GG	47(38.5)	47(42.7)	37(37.4)	20(40.0)	19(35.8)	18(37.5)	0.42(0.14-1.24)		0.23(0.06-0.81)		0.39(0.11-1.41)	
	G-carrier vs AA	108(88.5)	99(90.0)	88(88.9)	39(78.0)	42(79.2)	37(77.1)	0.38(0.14-1.03)	0.058	0.28(0.09-0.89)	0.029	0.39(0.12-1.28)	0.119
	GG vs A-carrier	47(38.5)	47(42.7)	37(37.4)	20(40.0)	19(35.8)	18(37.5)	0.93(0.44-1.96)	0.852	0.54(0.24-1.24)	0.142	0.77(0.31-1.88)	0.562
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	53(35.6)	48(32.7)	0.74(0.44-1.26)	0.266	0.52(0.29-0.95)	0.030	0.78(0.33-1.83)	0.221
rs960070	Codominant												
	GG	20(16.4)	16(14.5)	12(12.1)	12(24.0)	10(18.9)	10(20.8)	1.00 *	0.503	1.00 *	0.382	1.00 *	0.623
	CG	66(54.1)	58(52.7)	59(59.9)	24(48.0)	29(54.7)	23(47.9)	0.59(0.23-1.53)		0.78(0.25-2.40)		0.57(0.17-1.93)	
	CC	36(29.5)	36(32.7)	28(28.3)	14(28.0)	14(26.4)	15(31.2)	0.57(0.20-1.62)		0.46(0.13-1.59)		0.55(0.14-2.09)	
	C-carrier vs GG	102(83.6)	94(85.5)	87(87.9)	38(76.0)	43(81.1)	38(79.2)	0.58(0.24-1.43)	0.243	0.65(0.22-1.90)	0.431	0.56(0.17-1.81)	0.336
	CC vs G-carrier	36(29.5)	36(32.7)	28(28.3)	14(28.0)	14(26.4)	15(31.2)	0.83(0.37-1.85)	0.643	0.56(0.23-1.34)	0.188	0.85(0.33-2.18)	0.728
	Log-additive	122(80.8)	110(72.8)	99(66.0)	50(33.8)	53(35.6)	48(32.7)	0.77(0.45-1.31)	0.329	0.66(0.36-1.21)	0.178	0.77(0.40-1.49)	0.439

* reference estimate
** statistical significance retained after FDR correction

Haplotype analysis regarding professional and vocational dancers with normal BMD (reference group – dancers with normal BMD) and dancers with low BMD (Table 20; Appendix 7) showed an inverse association with low BMD at the FN in dancers with haplotype CG in *ESR1* [OR (CI)=0.53 (0.29-0.96), *p*=0.037]. Haplotype analysis also revealed that, within the same anatomical site, the odds of low BMD were significantly increased in dancers with the haplotype GCGT in *LRP5* [OR (CI)=8.97 (1.14-70.31), *p*=0.037]. Haplotype association tests considering normal controls (reference) and dancers with low BMD showed the CG haplotype in ESR1 was inversely associated with low BMD at the LS [OR (CI)=0.43 (0.22-0.82), *p*=0.001] and at the FN [OR (CI)=0.39 (0.19-0.80), *p*=0.010]. In *LRP5*, haplotype GCAG was significantly associated with low BMD at the forearm in dancers [OR (CI)=6.43 (1.33-31.14) *p*=0.021] and haplotype GCGT was associated with low BMD at the LS [OR (CI)=12.7 (1.22-132.18) *p*=0.033].

At baseline and at 1-yr follow-up, female vocational dancers revealed significantly higher sclerostin values than their controls (*p*<0.001) (Figure 10). There is no significant difference in serum sclerostin concentrations between male vocational dance students and their controls throughout the follow-up (*p*>0.05). Bone mass values were further adjusted for sex and sclerostin serum concentrations; after the adjustment, no differences in BMC and BMD between groups were seen at the FN and LS (*p*>0.05) (Table 21).



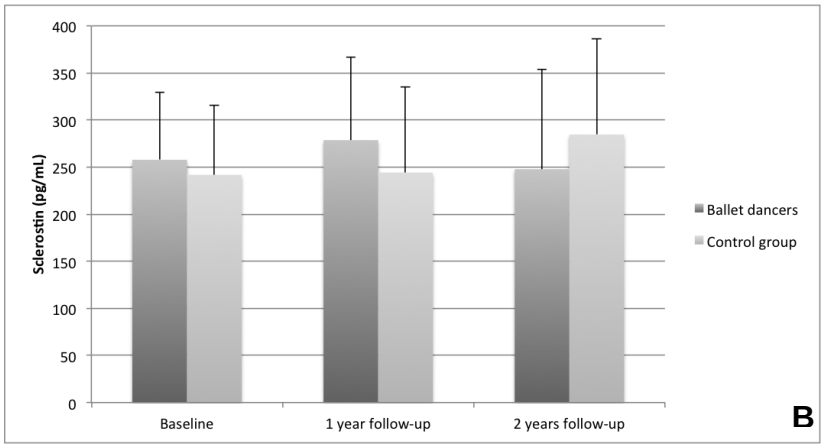


Figure 10. Serum levels of sclerostin throughout the 2-year follow up in female (A) and male (B) vocational dance students and aged-sex-matched controls

Table 21. Adjusted bone parameters for sex and sclerostin serum concentrations in vocational dance students

	Vocational dancers	IC 95%	Controls	IC 95%
FN measures				
BMC (g)	3.97 ± 0.16	3.64 – 4.29	4.26 ± 0.18	3.90 – 4.61
BMD (g/cm^2)	1.01 ± 0.03	0.94 – 1.07	1.03 ± 0.04	0.96 – 1.10
LS measures				
BMC (g)	40.6 ± 2.42	35.68 – 45.42	46.29 ± 2.58	41.11 – 51.47
BMD (g/cm^2)	0.92 ± 0.03	0.85 – 0.98	1.01 ± 0.03	0.94 – 1.07
FA measures				
BMC (g)	1.50 ± 0.06**	1.37 – 1.62	1.79 ± 0.06	1.67 – 1.91
BMD (g/cm^2)	0.62 ± 0.02***	0.58 – 0.66	0.76 ± 0.02	0.72 – 0.79

Values are means + SD
** p<0.01; *** p<0.001
BMC: bone mineral content; BMD: bone mineral density; FN: femoral neck; LS: lumbar spine; FA: forearm

DISCUSSION

Dance medicine data have suggested that dancers are at higher risk of developing low BMD and osteoporosis compared to the general population [18,25,43,146]. However, recent study from our group reinforced the fact that there is little evidence supporting the mechanisms and factors associated with low bone mass phenotypes in dancers [109]. Indeed, this study demonstrated that a relatively high number of professional dancers and vocational dance students were diagnosed with low BMD, but failed to identify any of the factors usually associated with bone mass in aesthetic athletes (i.e. body weight, menstrual disturbances, etc.); after the adjustment for these covariates, dancers with low BMD continued to display lower bone mass values at both impact and non-impact sites. Nevertheless, for the first time, it has been shown that some gene polymorphisms of the Wnt/β-catenin and ER signalling

pathways are significantly associated with low BMD in a cohort of professional ballet dance and vocational dance students. Specifically, the present study revealed that the *ESR1* rs9340799 A allele, *LRP5* rs2508836 C allele and *LRP5* GCGT/GCAG haplotypes are associated with an increased odds of low BMD in dancers at both impact (LS and FN) and non-impact sites (forearm). It was further found that serum sclerostin concentrations, a protein that inhibits bone formation by blocking the action of the Wnt in osteoblasts [166], was significantly higher in vocational dance students compared to non-exercise controls at all measured occasions. Taken together, these results suggest that the Wnt/β-catenin and ER signalling pathways are critical in determining dancers' bone mass phenotypes.

It is generally accepted that the low BMD displayed by elite dancers is associated with low body weight, menstrual disturbance or energy balance [11,24]. However, in previous studies of the present PhD thesis it has been shown that the aforementioned factors not always seem to be associated with BMD mass. Actually, previous studies of the current thesis were not able to find factors associated with BMD at impact sites, particularly at the LS (which is known to be more responsive to mechanical stress from exercise [10,133,156]). Basic science has been highlighting that the response to mechanical stress from exercise by osteoblast lineage cells requires full *ESR1* and *LRP5* activity [87,167–169]. Interestingly, the present study showed that dancers C homozygotes for SNP rs2508836 in *LRP5*, dancers *ESR1* rs9340799 A-carries, and dancers *LRP5* GCGT and GCAG haplotype-carriers have increased odds of developing low BMD at the LS (and also other anatomical sites). Therefore, it may be hypothesized that the degree to which each dancer responds to mechanical stress from dance training stimuli is associated with their genetic background. Indeed, observations with mice lacking functional *ESR1* have been showing that the osteogenic response to loading was reduced by 70% [167,168]. Moreover, it has also been pointed out that Lrp5 co-receptor is regulating osteoblasts activity following loading [87,170,171]. Therefore, because altered *ESR1* and *LRP5* play a role in the regulation of mechanical loading, it can be speculated *ESR1* rs9340799 and *LRP5* rs2508836 are modulating dancers' skeletal response to exercise. This hypothesis should be further investigated in RCT.

The functional significance of the studied SNPs and how they can influence gene expression remains to be elucidated. Both rs9340799 and rs2508836 are located in introns 1. First introns can affect gene transcription, polyadenylation,

mRNA export, translational efficiency, rate of mRNA decay, and also alter transcription factors binding sites [172–174]. Therefore, it could be speculated that one of these processes is influencing expression levels of *ESR1* and *LRP5*, and, consequently, susceptibility for low BMD phenotypes. Studies on other populations have also found associations between rs9340799 and rs2508836 and bone mass phenotypes [175–178]. Nevertheless, the mechanisms by which these SNPs may influence gene function and, ultimately, bone phenotypes, are unknown. Also, the possibility that the associations found in our study are due to linkage disequilibrium with others, causally associated, SNPs cannot be excluded.

Osteocytes mediate the osteogenic response from mechanical loading through the protein sclerostin (encoded by the SOST gene) [166]; SOST downregulation is associated with greater bone mass gains, whereas overexpression of SOST has been linked with low bone mass phenotypes [166,179]. In fact, intervention studies in humans showed that the levels of serum sclerostin decreases following exercise stimuli, resulting further in bone mass gains [180,181]. Considering these findings, it would be expected to find in vocational dance students significantly lower sclerostin concentrations and higher bone mass values compared to controls as they progress on their training and receive osteogenic activity. Interestingly, dancers showed significantly higher sclerostin levels and lower BMD levels than controls. However, after the adjustment for sclerostin, vocational dance students revealed higher bone mass values at both impact ad non-impact sites. As sclerostin is a key protein in Wnt/β-catenin, this reinforces that this pathway might be a fundamental in determining bone mass phenotypes in dancers.

One limitation of the present study is population stratification, a characteristic common in most genetic association studies. Considering the number of dancers (n=151) and age and sex-matched controls genotyped (n=151), the present study has over 85% power to detect modest genetic effect (OR of 2.0 and MAF=0.2). However, the present study looses some power after stratification. The lack of association with other SNPs might also be due to stratification; GWAS should be considered in future studies. An additional limitation might be the fact that searching for the association of several SNPs with more than one phenotype can lead to a type I error, i.e. the possibility of false-positive associations increase. To avoid this limitation a multiple test correction (FDR) was applied. Moreover, in a genetic association study, ideally, participants should be matched for variables such as age,

body weight, sex, and other potential confounders to avoid bias. It was matched dancers with controls of the same age and sex, however, the matching was lost after stratification. It can also be assumed that another weakness of this study is that both male and female dancers were included in the same group. Both sexes were included in the same group because there is some evidence showing that the distribution of Esr1 receptors in bone cells is similar among sexes [182,183]. Therefore, the impact of this potential limitation may not be critical.

CONCLUSION

Genetic variants at the Wnt/β-catenin and ER signalling pathways are potential risk factors for low BMD in professional dancers and vocational dance students. The involvement of these pathways in dancers' bone mass pathogenesis needs further investigation at both basic and clinical level.

CHAPTER 10: GENERAL DISCUSSION

Principal findings

The conventional position on dancers' bone health is that these professionals are at increased risk for low BMD and osteoporosis in later life due to low body weight, menstrual disturbances and/or low energy availability for performance. The present PhD thesis aimed at a further understanding of the factors associated with BMD in this population. This PhD thesis shows that the aforementioned factors not always seem to be associated with BMD in professional and vocational dance students. Thus, it can be hypothesised that other mechanism rather than the GH – IGF-1 axis and HHG axis might be involved in determining dancers' bone health. Actually, the results of the present study point to the direction that our dancers may be exhibiting low BMD due to a mechanism related to mechanotransduction, whereas the Wnt/β-catenin and ER seem to be key pathways for our dancers' bone health, since:

1. Even before starting the vocational dance training, first year female vocational dance students already revealed lower bone health status and lower body weight compared to non-exercising controls (Study 1);

2. Body weight and fat mass were significantly reduced in dancers compared to controls, but these factors were not associated with bone mass (Study 2);

3. When exploring further these findings on a longitudinal analysis it was found that, as vocational dancers receive osteogenic stimuli (dance training) throughout time, their bone mass remained consistently lower compared to non-exercising controls. Traditional osteoporosis risk factors were not able to predict group differences in terms of bone mass gains throughout time at all anatomical sites (Studies 3 and 4);

4. In vocational dance students, energy availability lied within the normal range throughout three years of follow-up (Study 4);

5. Longitudinal serum IGF-1 concentrations (a potential marker of hypothalamic dysfunction) were similar or significantly higher in vocational dancers than controls (Studies 3 and 4);

6. A relatively high number of professional ballet dancers and vocational dance students were diagnosed with low BMD, but did not displayed any traditional osteoporosis risk factors (and vice-versa) (Study 5);

7. After cross-sectional adjustment for traditional osteoporosis risk factors, dancers with low BMD continued to display lower bone mass values than dancers with normal BMD at both impact (LS and FN), and non-impact sites (forearm) (Study 5);

8. Instead, serum sclerostin concentrations (a potential mechanotransduction marker) were significantly increased throughout the follow-up in vocational dance students compared to controls, and, after the adjustment for this covariate, differences between groups in BMD were dissipated at all anatomical sites (Study 5);

9. Genetic variants at the Wnt/β-catenin and ER signalling pathways (mechanotransduction pathways) were found to be risk factors for low BMD in dancers at both impact and non-impact sites (Study 5);

Other studies also found that athletic populations consistently exhibit low bone mass parameters compared to non-exercising populations throughout a 3-yr follow-up [157]; and that low BMD not always is associated with body weight, menstrual disturbance and/nor energy balance [48,109,184–188]. However, these reports do not give explanation for their findings. Consequently, low BMD in these athletes remain apparently unexplained, as no other mechanism apart from the GH – IGF-1 axis and HHG axis has been proposed to be associated with low BMD in athletes.

Possible mechanisms

If the GH – IGF-1 axis and HHG axis were playing a significant role in determining bone mass phenotypes in our dancers, it would be expected to find significantly lower IGF-1 serum concentrations in our exercise population compared to controls, as it has been documented that IGF-1 is sensitive to changes in the hypothalamus [140]. This hormone was not altered in our vocational dancers, and its concentrations significantly increased as female dancers progress on their training, which may indicate an effect of exercise since IGF-1 serum concentration is known to be increased in athletes [140,150]. However, it has been highlighted that the response

to mechanical stress from exercise by bone cells requires full activity of ER and Wnt/β-catenin signalling pathways [132,155]. Wnt regulates osteoblasts activity through the β-catenin molecule [189]. Wnt/β-catenin is activated by binding to one of its co-receptors, the low-density lipoprotein receptor-related protein 5 (*LRP5*, encoded by the *LRP5* gene). The result is a signalling cascade, ultimately leading to a stimulation of osteoblasts activity [87]. Moreover, the oestrogen receptor alpha plays a critical role in translocating β-catenin into the nucleus in response to mechanical loading [87]. Interactions between Wnt/β-catenin and ER signalling pathways have only recently been described [179], but it is generally accepted that the response to mechanical stimuli from exercise (i.e. mechanotransduction) by osteoblast lineage cells requires full activity of the aforementioned pathways [87,167,168,170]. In accordance, genes of the Wnt/β-catenin and ER signalling pathways (e.g. *SOST*, *LRP5*, *ESR1* and *ESR2*) may be clinically important and may play a significant role in mediating the response to mechanical stress from exercise [170,177]. Actually, as it was found that dancers C homozygotes for SNP rs2508836 in *LRP5* and dancers *ESR1* rs9340799 A-carriers have increased odds of developing low BMD at both impact and non-impact sites, it can be hypothesize that the degree to which our dancers respond to mechanical stimuli from exercise is associated with their genetic background. However, as previously mentioned (Study 5) the functional significance of the aforementioned SNPs and how they can influence gene expression remains to be elucidated. It could be speculated that these SNPs are influencing expression of *ESR1* and *LRP5* in our dancers, and, consequently, susceptibility for low BMD phenotypes. Furthermore, LRP5 co-receptor activity is inhibited by the Wnt antagonist sclerostin, a glycoprotein encoded by the *SOST* gene [189]; *SOST* overexpression causes low bone mass phenotypes in humans [166]. It has been showed that *SOST* downregulation is required for osteogenic response to mechanical loading stimuli [166]. Studies on animal models have showed that sclerostin protein levels of mice were significantly reduced following loading [190,191]. In humans, serum sclerostin concentrations were also found to be significantly decreased (and IGF-1 significantly increased) following both resistance and jumping exercise intervention programmes [180,181]. Interesting, the longitudinal vocational dancers subgroup revealed significantly higher serum sclerostin concentrations compared to non-exercise controls in all measure occasions. Because our dance population was involved in daily exercise sessions and several

hours of weight-bearing activity, we were expecting to find significantly lower serum sclerostin concentrations compared to controls following *SOST* downregulation in response to the dance training [166]. Interesting though, in a sample of artistic gymnast, sclerostin and preadipocyte factor-1 (hormone that also inhibit bone formation) were found to be higher; however, gymnasts' bone mass was higher as compared to controls [192]. Although no studies reporting the existence of an exercise stimuli threshold above which bone cells saturate were found, this hypothesis should not be excluded. Future studies should explore *SOST* and sclerostin activity in elite dancers and other athletes.

It should be noted that it is very unlikely that low BMD phenotypes are inherited in a Mendelian manner, since low BMD onset and progression are influenced by genetic factors modulated by environmental factors [152]. Actually, the results of this Thesis may express this view. Comparing controls with normal BMD (not receiving exercise stimuli) to dancers with low BMD (receiving exercise stimuli), it was observed that *ESR1* rs9340799 A allele and *LRP5* rs2508836 C allele are associated with an increased odds of low BMD in dancers, not only at non-impact sites, but also at impact sites. In turn, comparing dancers with normal BMD with dancers with low BMD (both groups receiving exercise stimuli), only *ESR1* rs9340799 A allele was associated with an increased odds of low BMD at non-impact sites, and not at impact sites. Since the associations between the risk alleles and phenotypes at impact sites are not manifested when comparing dancers with normal BMD and dancers with low BMD (both groups receiving exercise stimuli), one could thus assume that genetic and environmental factors are interacting tightly in determining dancers' bone mass phenotypes. Indeed, this finding might reflect gene-environment interaction; randomized control trials are needed to confirm this hypothesis.

Implications for clinicians and national health systems

The results of this PhD thesis raise concerns regarding the current screening and intervention guidelines for aesthetic athletes' bone health. Clinicians should be aware that even dancers that do not have any sign of traditional osteoporosis risk factors might have low BMD. Indeed, study 5 revealed that based on current screening guidelines [193], physicians would not have been able to detect a significant number of dancers with low BMD. Furthermore, physicians usually focus on the treatment by

restoring menses, increasing energy availability, nutritional education and body weight gain [194–196]. However, focusing on the spectrum of energy availability, low body weight and low BMD, many dancers may not receive adequate diagnosis or treatment. Actually, because there has been a large emphasis on the treatment of low BMD in athletes through restoring hypothalamus function, treatments such as hormone therapy and oral contraceptives are usually recommended for these patients [57,197]. However, those have been showed to be inefficient to restore dancers' bone mass [57] (as well as other female athletes' bone mass [196]), which suggests that other mechanisms may be involved in shaping dancers' bone mass.

The findings of the present study may have serious clinical implications, as young elite dancers may enter adulthood with low BMD. If dancers at risk are not accurately detected and monitored during growth, the peak bone mass may be impaired, increasing the odds for osteoporosis later in life and susceptibility to fracture [3]. These may have consequences not only for one's wellbeing, but also for the national health system. In 2010, UK health system spent 5.408,00 million euros in osteoporosis treatment, plus 33.756,00 euros in nursing home costs [119]. The number of deaths related to fracture has been estimated to be 26.256,00, and the unit costs for osteoporosis treatment and management 483,00 euros per patient (this estimate includes DXA scan, physician visit, annual generic pharmacological treatment and long-term costs; if treatment is different than generic alendronate, the cost increases) [119]. Indirect costs such as reduced productivity while at work, inability to work and lost productive capacity due to fractures has not been taken into consideration on these estimations. Out of the 151 dancers screened in the present study, 80 had low BMD at least in one anatomical site, which means that around 50% of our population were disease positive. We were not able to find the exact number of elite dancers performing in Portugal and/or UK, but in a hypothetical sample of 10.000 professional and vocational dancers, around 1.712 would not be detected as disease positive. If such number is maintained into adulthood, the costs to treat these dancers could be around 826.896,00 euros in the future. Furthermore, as the present Thesis shows that not always the traditional osteoporosis risk factors are associated with low BMD, we do not know how many out of those detected as disease positive will be able to restore bone mass values following current treatment guidelines; if dancers are not accurately treated, the costs for will be increased. Considerations in relation to the best treatment for our dancers with low BMD is beyond the aim of the

present PhD thesis, but according to our main finding, it seems that the current guidelines treatment might not be appropriate [193–196]. However, genetic testing and personalised medicine is a topic under debate. Further, to current author's knowledge, research on sclerostin has not proposed normative values for circulating levels of this protein. Anti-sclerostin antibodies are currently under Phase III clinical trials, and it is believed that anti-sclerostin therapeutics will be promising for low BMD treatment [181]. Nevertheless, until more research on the topic is not done, it is recommended that all dancers involved in elite dancing should be referred for densitometry, including male participants.

CHAPTER 11: STRENGTHS, LIMITATIONS AND FUTURE RESEARCH

The results of the present study are unique and of relevance as the majority of previous studies on dancers' bone health have been mainly based on cross-sectional designs, whereas only a small number of osteoporosis risk factors are measured in the same population of female dancers. The present work incorporates both cross-sectional and longitudinal designs within the same population, and several factors (genetics, endocrinology, anthropometry, nutrition, energy availability and traditional osteoporosis risk factors) have been considered simultaneously. Genetic factors and bone health in male dancers have not been previously considered.

A limitation of the present study is that the sample might not reflect the entire population of elite dancers. Further, mixed longitudinal designs do not allow a cause-effect, and potential confounding factors cannot be fully controlled. Hormone serum concentrations were only investigated in vocational dancers and not in professional ballet dancers; it would be interesting for future studies to investigate the incidence of low BMD and risk factors in professional dancers, sclerostin serum concentrations and IGF-1. The assessment of bone formation and resorption markers (in both professional and vocational dancers) would also be interesting to consider in future research. The longitudinal comparison of bone mass parameters between dancers from Portugal and UK it would further give insights into the modifiable factors (i.e. nutrition, energy expenditure, energy availability and dance training regiments) associated with bone mass accruals in groups under the influence of the same aesthetic regiments. Also, fractures or injuries among our population of dancers were not recorded. In order to establish the clinical significance of our findings, it would be of great interest that future studies provide information on the association between low BMD with dance injuries and/or fractures. The main outcome in this PhD thesis – BMC and BMD, measured by DXA - does not account for changes in bone microarchitecture; however, this device is considered the "gold standard" to longitudinally measure bone mass, even in children [158]. The use of two different DXA scans to assess participants and the need to adjust the data for potential bias is a limitation. However, this approach has been previously used [101,102]. Nevertheless, to further substantiate the findings of this PhD thesis, it is recommended the assessment of bone microarchitecture, a well-known determinant of bone strength. Indeed, future studies are needed to validate our results.

Determination of the exact mechanisms involved in dancers' bone health can only be obtained by studies investigating simultaneously the molecular pathways involved in mechanotransduction, and the GH – IGF-1 axis and HHG axis within a larger sample of dancers. Training volume and intensity should also be considered, as well as the epigenetic effects of athletic training in bone cells function. The present of the female athlete triad and/or RED-S in elite dancing should also be further investigating as, to the best of my knowledge, there is not evidence of the presence of these syndromes in dancers. Actually, the mechanisms related to the triad and the presence of triad as been questioned [23,188]. If the results of the present study are confirmed in future studies, these can have a significant impact on the implementation of screening and intervention programs in dancers.

CHAPTER 12: GENERAL CONCLUSIONS

Both professionals and vocational dance students (female and male) have lower bone health compared to general population. Apart form the significant reduced body weight, clinicians and researchers should be aware that other mechanisms apart from the ones usual considered (i.e. the GH – IGF-1 axis and HHG axis) might be involved in determining dancers' bone mass. Genetic variants at the Wnt/β-catenin and ER signalling pathways are potential risk factors for low BMD in elite dancers. The involvement of these pathways in dancers' bone mass pathogenesis (both in female and male participants) needs further investigation at both basic and clinical level, as well as the role of the protein sclerostin. Nevertheless, until more research is done, it is recommend that dancers performing at elite level should be referred for bone densitometry.

In summary:

What was known in this topic:

- Bone health is a concern among dancers
- Conventional position on dancers' bone health is that female participants are at increased risk for low BMD and osteoporosis in later life in the presence of low body weight, menstrual disturbances and/or low energy availability
- Mechanisms related to the GH – IGF-1 and HHG axis are involved in dancers' bone health
- Less is known about male dancers

What this PhD thesis adds:

- Factors traditional associated with BMD in dancers (i.e. body weight, menstruation and energy availability) do not fully explain the low bone mass values displayed by dancers
- Other mechanisms rather than the GH – IGF-1 and HHG axis might be involved in dancers' bone health
- Genetic variants at the Wnt/β-catenin and ER signalling pathways are potential risk factors for low BMD in dancers
- Male dancers might also be at risk for low BMD

CHAPTER 13: REFERENCES

1. Xu J, Lombardi G, Jiao W, Banfi G. Effects of Exercise on Bone Status in Female Subjects, from Young Girls to Postmenopausal Women: An Overview of Systematic Reviews and Meta-Analyses. Sports Med. [Internet]. Springer International Publishing; 2016; Available from: http://link.springer.com.libproxy.furman.edu/article/10.1007/s40279-016-0494-0/fulltext.html
2. Robson H, Siebler T, Shalet SM, Williams GR. Interactions between GH, IGF-I, glucocorticoids, and thyroid hormones during skeletal growth. Pediatr. Res. 2002;52:137–47.
3. Heaney RP, Abrams S, Dawson-Hughes B, Looker A, Marcus R, Matkovic V, et al. Peak bone mass. Osteoporos. Int. 2000;11:985–1009.
4. Lorentzon M, Cummings SR. Osteoporosis: The evolution of a diagnosis. J. Intern. Med. 2015;277:650–61.
5. Kanis JA, Johnell O. Requirements for DXA for the management of osteoporosis in Europe. Osteoporos. Int. 2005;16:229–38.
6. Karaguzel G, Holick MF. Diagnosis and treatment of osteopenia. Rev. Endocr. Metab. Disord. 2010;11:237–51.
7. Cosman F, Lindsay R, LeBoff MS, Jan de Beur S, Tanner B. National Osteoporosis Foundation. Clinician's Guide to Prevention and Treatment of Osteoporosis. Washington, DC: National Osteoporosis Foundation; 2014. Natl. Osteoporos. Found. 2014;1:55.
8. Hind K, Burrows M. Weight-bearing exercise and bone mineral accrual in children and adolescents: A review of controlled trials. Bone. 2007;40:14–27.
9. Nurmi-Lawton JA, Baxter-Jones AD, Mirwald RL, Bishop JA, Taylor P, Cooper C, et al. Evidence of Sustained Skeletal Benefits From Impact-Loading Exercise in Young Females: A 3-Year Longitudinal Study. J. Bone Miner. Res. [Internet]. 2003;19:314–22. Available from: http://doi.wiley.com/10.1359/JBMR.0301222
10. Guadalupe-Grau A, Fuentes T, Guerra B, Calbet JAL. Exercise and bone mass in adults. Sport. Med. [Internet]. 2009;39:439–68. Available from: http://search.ebscohost.com/login.aspx?direct=true&db=jlh&AN=2010312904&site=ehost-live
11. Nattiv A, Loucks AB, Manore MM, Sanborn CF, Sundgot-Borgen J, Warren MP. The female athlete triad. Med. Sci. Sports Exerc. 2007;39:1867–82.
12. Gibbs JC, Williams NI, De Souza MJ. Prevalence of individual and combined components of the female athlete triad. Med. Sci. Sports Exerc. 2013;45:985–96.
13. Koutedakis Y, Jamurtas AZ. The dancer as a performing athlete. Sport. Med. 2004;34:651–61.
14. Twitchett E, Angioi M, Koutedakis Y, Wyon M. Video analysis of classical ballet performance. J. Dance Med. Sci. [Internet]. 2009;13:124–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22052033
15. Bennell, Kim; Khan, Karim; Matthews, Bernadette; Cook, Elizabeth; Holzer, Karen; McKay, Heather; Wark J. Activity-Associated differences in bone mineral are evident before puberty.pdf. Pediatr. Exerc. Sci. 2000;12:371–81.
16. Yannakoulia M, Keramopoulos A, Matalas AL. Bone mineral density in young active females: The case of dancers. Int. J. Sport Nutr. Exerc. Metab. 2004. p. 285–97.
17. Robson B, Chertoff A. Bone Health and Female Dancers: Physical and Nutritional Guidelines. Int. Assoc. Danc. Med. Sci. [Internet]. 2010;1–4. Available from:

http://c.ymcdn.com/sites/www.iadms.org/resource/resmgr/resource_papers/bone_health_female_dancers.pdf
18. Doyle-Lucas AF, Akers JD, Davy BM. Energetic efficiency, menstrual irregularity, and bone mineral density in elite professional female ballet dancers. J. Dance Med. Sci. 2010;14:146–54.
19. Burckhardt P, Wynn E, Krieg M-A, Bagutti C, Faouzi M. The effects of nutrition, puberty and dancing on bone density in adolescent ballet dancers. J. Dance Med. Sci. 2011;15:51–60.
20. Georgopoulos N a, Markou KB, Theodoropoulou A, Vagenakis G a, Mylonas P, Vagenakis AG. Growth, pubertal development, skeletal maturation and bone mass acquisition in athletes. Hormones (Athens). 2004;3:233–43.
21. Wheatley S, Khan S, Székely AD, Naughton DP, Petróczi A. Expanding the Female Athlete Triad concept to address a public health issue. Perform. Enhanc. Heal. [Internet]. Elsevier Ltd; 2012;1:10–27. Available from: http://dx.doi.org/10.1016/j.peh.2012.03.001
22. Bonjour JP, Chevalley T. Pubertal timing, bone acquisition, and risk of fracture throughout life. Endocr. Rev. 2014;35:820–47.
23. Di Pietro L, Stachenfeld N. Refutation of the myth of the female athlete triad. Br. J. Sports Med. [Internet]. 2006;41:57–8. Available from: http://bjsm.bmj.com/cgi/doi/10.1136/bjsm.2006.032854
24. Mountjoy M, Sundgot-Borgen J, Burke L, Carter S, Constantini N, Lebrun C, et al. The IOC consensus statement: beyond the Female Athlete Triad--Relative Energy Deficiency in Sport (RED-S). Br. J. Sports Med. [Internet]. 2014;48:491–7. Available from: http://bjsm.bmj.com/content/48/7/491.abstract
25. Keay N, Fogelman I, Blake G. Bone mineral density in professional ballet dancers. Bone Miner. 1997;31:143–7.
26. Yang LC, Lan Y, Hu J, Yang YH, Zhang Q, Huang ZW, et al. Relatively high bone mineral density in Chinese adolescent dancers despite lower energy intake and menstrual disorder. Biomed. Environ. Sci. 2010;23:130–6.
27. To W, Wong M. Does oligomenorrhoea/amenorrhoea and underweight imply athlete triad syndrome in young female dancers? Eur. J. Sport Sci. [Internet]. 2011;11:335–40. Available from: http://search.ebscohost.com/login.aspx?direct=true&db=s3h&AN=65143564&site=ehost-live&scope=site
28. Young N, Formica C, Szmukler G, Seeman E. Bone density at weight-bearing and non weight bearing sites in ballet dancers: the effects of exercise, hypogonadism, and body weight. J. Clin. Endocrinol. Metab. 1994;78:449–54.
29. Karlsson MK, Johnell O, Obrant KJ. Bone mineral density in professional ballet dancers. Br. J. Sports Med. 1997;31:143–7.
30. Mitchell BD, Yerges-Armstrong LM. The genetics of bone loss: Challenges and prospects. J. Clin. Endocrinol. Metab. 2011;96:1258–68.
31. Ralston SH, Uitterlinden AG. Genetics of Osteoporosis. Endocr. Rev. [Internet]. 2010;31:629–62. Available from: http://press.endocrine.org/doi/abs/10.1210/er.2009-0044
32. Cummings SR, Browner W, Cummings SR, Black DM, Nevitt MC, Browner W, et al. Bone density at various sites for prediction of hip fractures. Lancet. 1993;341:72–5.
33. Melton LJ, Atkinson EJ, O'Fallon WM, Wahner HW, Riggs BL. Long-term fracture prediction by bone mineral assessed at different skeletal sites. J. Bone Miner. Res. 1993;8:1227–33.

34. Kralll E a, Dawson-Hughes BD a. Heritable and life-style determinants of bone mineral. J. Bone Miner. Res. 1993;8.
35. Naganathan V, Macgregor A, Snieder H, Nguyen T, Spector T, Sambrook P. Gender differences in the genetic factors responsible for variation in bone density and ultrasound. J. Bone Miner. Res. [Internet]. 2002;17:725–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11918230
36. G V-R. How does exercise affect bone development during growth? Sport. Med. 2006;36:561–9.
37. Wallace B a, Cumming RG. Systematic review of randomized trials of the effect of exercise on bone mass in pre- and postmenopausal women. Calcif. Tissue Int. 2000;67:10–8.
38. Snow-Harter C, Bouxsein ML, Lewis BT, Carter DR, Marcus R. Effects of resistance and endurance exercise on bone mineral status of young women: A randomized exercise intervention trial. J. Bone Miner. Res. 2009;7:761–9.
39. Lieberman DE. Adaptive skeletal responses to mechanical loading during adolescence. Sport. Med [Internet]. 2006;36:723–32. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19062774
40. Calbet JAL, Moysi JS, Dorado C, Rodriguez LP. Bone mineral content and density in professional tennis players. Calcif. Tissue Int. 1998;62:491–6.
41. Castelo-Branco C, Reina F, Montivero AD, Colodrón M, Vanrell JA. Influence of high-intensity training and of dietetic and anthropometric factors on menstrual cycle disorders in ballet dancers. Gynecol. Endocrinol. [Internet]. 2006;22:31–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16522531
42. Vescovi JD, Jamal SA, De Souza MJ. Strategies to reverse bone loss in women with functional hypothalamic amenorrhea: A systematic review of the literature. Osteoporos. Int. 2008;19:465–78.
43. Valentino R, Savastano S, Tommaselli AP, D'Amore G, Dorato M, Lombardi G. The influence of intense ballet training on trabecular bone mass, hormone status, and gonadotropin structure in young women. J. Clin. Endocrinol. Metab. 2001;86:4674–8.
44. Armann S, Wells C, Cheung S. Bone mass, menstrual abnormalities, dietary intake, and body composition in classical ballerinas. Kinesiol. Med. Danc. 13:1–15.
45. Pearce G, Bass S, Young N, Formica C, Seeman E. Does weight-bearing exercise protect against the effects of exercise-induced oligomenorrhea on bone density? Osteoporos. Int. 1996;6:448–52.
46. Waren, Michelle P; Brooks-Gunn, J; Fox, Richard P; Lancelot, Cynthia; Newman, Denise; Hamilton WG. Lack of bone accretion and amenorrhea: evidence for a relative osteopenia in wieght-bearing bones. J. Clin. Endocrinol. Metab. 1991;72:847–53.
47. van Marken Lichtenbelt WD, Fogelholm M, Ottenheijm R, Westerterp KR. Physical activity, body composition and bone density in ballet dancers. Br. J. Nutr. 1995;74:439–51.
48. Khan KM, Green RM, Saul a, Bennell KL, Crichton KJ, Hopper JL, et al. Retired elite female ballet dancers and nonathletic controls have similar bone mineral density at weightbearing sites. J. Bone Miner. Res. 1996;11:1566–74.
49. Slavin RE. Best evidence synthesis: an intelligent alternative to meta-analysis. J. Clin. Epidemiol. 1995;48:9–18.
50. Guyatt GH, Oxman AD, Vist GE, Kunz R, Falck- Y, Alonso-coello P, et al. Rating Quality of Evidence and Strength of Recommendations : GRADE : An Emerging

Consensus on Rating Quality of Evidence and Strength of Recommendations RATING QUALITY OF EVIDENCE OF RECOMMENDATIONS GRADE : of evidence an emerging and consensus of on rati. Br. Med. J. 2008;336:924–6.
51. Moher D, Liberati A, Tetzlaff J, Altman DG, Grp P. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. Phys. Ther. 2009;89:873–80.
52. Hoch AZ, Papanek P, Szabo A, Widlansky ME, Schimke JE, Gutterman DD. Association between the female athlete triad and endothelial dysfunction in dancers. Clin. J. Sport Med. 2011;21:119–25.
53. Bass S, Pearce G, Young N. Bone mass during growth: the effects of exercise. Acta Univ Carol Med. 1994;40:3–6.
54. To WWK, Wong MWN, Lam IYL. Bone mineral density differences between adolescent dancers and non-exercising adolescent females. J. Pediatr. Adolesc. Gynecol. 2005;18:337–42.
55. Kaufman BA, Warren MP, Dominguez JE, Wang J, Heymsfield SB, Pierson RN. Bone density and amenorrhea in ballet dancers are related to a decreased resting metabolic rate and lower leptin levels. J. Clin. Endocrinol. Metab. 2002;87:2777–83.
56. Quintas ME, Ortega RM, López-Sobaler a M, Garrido G, Requejo a M. Influence of dietetic and anthropometric factors and of the type of sport practised on bone density in different groups of women. Eur. J. Clin. Nutr. 2003;57 Suppl 1:S58–62.
57. Warren MP, Brooks-Gunn J, Fox RP, Holderness CC, Hyle EP, Hamilton WG, et al. Persistent osteopenia in ballet dancers with amenorrhea and delayed menarche despite hormone therapy: A longitudinal study. Fertil. Steril. 2003;80:398–404.
58. Wolman RL, Faulmann L, Clark P, Hesp R, Harries MG. Different training patterns and bone mineral density of the femoral shaft in elite, female athletes. 1991;50:487–9. Available from: pm:1877854
59. Kilicarslan A, Isildak M, Guven GS, Oz SG, Hasbay A, Karabulut E, et al. The influence of ballet training on bone mass in Turkish ballet dancers. Endocrinologist. 2007;17:85–8.
60. Frederick L, Hawkins S. A comparison of knowledge and attitudes, dietary practices, and bone densities of postmenopausal women, female college athletes, and nonathletic college women. J Am Diet Assoc. 1992;93:299–305.
61. Muñoz Calvo MT, de la Piedra C, Barrios V, Garrido G, Argente J. Changes in bone density and bone markers in rhythmic gymnasts and ballet dancers: Implications for puberty and leptin levels. Eur. J. Endocrinol. 2004;151:491–6.
62. Hinrichs T, Chae E-H, Lehmann R, Allolio B, Platen P. Bone Mineral Density in Athletes of Different Disciplines: a Cross- Sectional Study. Open Sports Sci. J. 2010;3:129–33.
63. Friesen KJ, Rozenek R, Clippinger K, Gunter K, Russo AC, Sklar SE. Bone mineral density and body composition of collegiate modern dancers. J. Dance Med. Sci. [Internet]. 2011;15:31–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21703091
64. Cuesta A, Revilla M, Villa LF, Hernández ER, Rico H. Total and regional bone mineral content in Spanish professional ballet dancers. Calcif. Tissue Int. 1996;58:150–4.
65. Foldes AJ, Danziger A, Constantini N, Popovtzer MM. Reduced ultrasound velocity in tibial bone of young ballet dancers. Int. J. Sports Med. 1997;18:296–9.
66. Tsai SC, Hsu HC, Fong YC, Chiu CC, Kao A, Lee CC. Bone mineral density in young female Chinese dancers. Int. Orthop. 2001;25:283–5.
67. Matthews BL, Bennell KL, McKay HA, Khan KM, Baxter-Jones ADG, Mirwald RL,

et al. Dancing for bone health: A 3-year longitudinal study of bone mineral accrual across puberty in female non-elite dancers and controls. Osteoporos. Int. 2006;17:1043–54.
68. Yang L-C, Lan Y, Hu J, Yang Y-H, Zhang Q, Piao J-H. Correlation of serum leptin level with bone mineral density and bone turnover markers in Chinese adolescent dancers. Biomed. Environ. Sci. [Internet]. 2009;22:369–73. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20163060
69. Oral A, Tarakçi D, Disci R. Calcaneal quantitative ultrasound measurements in young male and female professional ballet dancers. Strength Cond Res. 2006;20:572–8.
70. Warren MP, Brooks-Gunn J, Fox RP, Holderness CC, Hyle EP, Hamilton WG. Osteopenia in exercise-associated amenorrhea using ballet dancers as a model: A longitudinal study. J. Clin. Endocrinol. Metab. 2002;87:3162–8.
71. Khan KM, Bennell KL, Hopper JL, Flicker L, Nowson C a, Sherwin a J, et al. Self-reported ballet classes undertaken at age 10-12 years and hip bone mineral density in later life. Osteoporos. Int. 1998;8:165–73.
72. Myburgh K, Hutchins J, Fataar A, Hough S, Noakes T. Low Bone Density Is an Etiologic Factor forStress Fractures in Athletes. Ann Intern Med. 1990;113:754–9.
73. Lauder TD, Dixit S, Pezzin LE, Williams M V., Campbell CS, Davis GD. The relation between stress fractures and bone mineral density: Evidence from active-duty army women. Arch. Phys. Med. Rehabil. 2000;81:73–9.
74. Keen AD, Drinkwater BL. Irreversible bone loss in former amenorrheic athletes. Osteoporos. Int. 1997;7:311–5.
75. Bailey D a. The Saskatchewan Pediatric Bone Mineral Accrual Study: bone mineral acquisition during the growing years. Int. J. Sports Med. [Internet]. 1997;18 Suppl 3:S191-4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9272847
76. Baxter-Jones ADG, Faulkner RA, Forwood MR, Mirwald RL, Bailey DA. Bone mineral accrual from 8 to 30 years of age: An estimation of peak bone mass. J. Bone Miner. Res. 2011;26:1729–39.
77. Davies JH, Evans B a J, Gregory JW. Bone mass acquisition in healthy children. Arch. Dis. Child. 2005;90:373–8.
78. Specker B, Schoenau E. Quantitative bone analysis in children: current methods and recommendations. J Pediatr. 2005;146:726–31.
79. Maïmoun L, Sultan C. Effects of physical activity on bone remodeling. Metabolism. [Internet]. Elsevier B.V.; 2011;60:373–88. Available from: http://dx.doi.org/10.1016/j.metabol.2010.03.001
80. Ausili E, Rigante D, Salvaggio E, Focarelli B, Rendeli C, Ansuini V, et al. Determinants of bone mineral density, bone mineral content, and body composition in a cohort of healthy children: Influence of sex, age, puberty, and physical activity. Rheumatol. Int. 2012;32:2737–43.
81. Bachrach LK, Hastie T, Wang M, Narasimhan B, Marcus R, B DPLK, et al. Bone Mineral Acquisition in Healthy Asian , Hispanic , Black , and Caucasian Youth : A Longitudinal Study *. 1999;84:4702–12.
82. Koutedakis Y, Sharp N. Thigh-muscles strength training, dance exercise, dynamometry, and anthropometry in professional ballerinas. J Strength Cond Res. 2004;18:714–718.
83. Koutedakis Y, Hukam H, Metsios G, Nevill A, Giakas G, Jamurtas A, et al. The effects of three months of aerobic and strength training on selected performance- and fitness-related parameters in modern dance students. Strength Cond. 2007;21:808–12.

84. Allen N, Nevill A, Brooks J, Koutedakis Y, Wyon M. Ballet Injuries: Injury Incidence and Severity Over 1 Year. J. Orthop. Sports Phys. Ther. 2012;42:781–90.
85. Allen N, Nevill AM, Brooks JHM, Koutedakis Y, Wyon MA. The effect of a comprehensive injury audit program on injury incidence in ballet: a 3-year prospective study. Clin. J. Sport Med. 2013;23:373–8.
86. Koutedakis Y, Khaloula M, Pacy PJ, Murphy M, Dunbar GMJ. Thigh Peak Torques and Lower-Body Injuries in Dancers. J. Danc. Med. Sci. 1997;1:12–5.
87. Bonewald LF, Johnson ML. Osteocytes, mechanosensing and Wnt signaling. Bone. 2008;42:606–15.
88. Morris NM, Udry JR. Validation of a self-administrated instrument to assess stage of adolescent development. J. Youth Adolesc. 1980;9:271–280.
89. Mirwald RL, Baxter-Jones ADG, Bailey D a, Beunen GP. An assessment of maturity from anthropometric measurements. Med. Sci. Sports Exerc. [Internet]. 2002;34:689–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11932580
90. Crawford PB, Obarzanek E, Morrison J, Sabry ZI. Comparative advantage of 3-day food records over 24-hour recall and 5-day food frequency validated by observation of 9- and 10-year-old girls. J. Am. Diet. Assoc. 1994;94:626–630.
91. Brazeau AS, Beaudoin N, Bélisle V, Messier V, Karelis AD, Rabasa-Lhoret R. Validation and reliability of two activity monitors for energy expenditure assessment. J. Sci. Med. Sport [Internet]. Sports Medicine Australia; 2016;19:46–50. Available from: http://dx.doi.org/10.1016/j.jsams.2014.11.001
92. Weeks BK, Beck BR. The BPAQ: A bone-specific physical activity assessment instrument. Osteoporos. Int. 2008;19:1567–77.
93. Cardadeiro G, Baptista F, Rosati N, Zymbal V, Janz KF, Sardinha LB. Influence of physical activity and skeleton geometry on bone mass at the proximal femur in 10- to 12-year-old children - A longitudinal study. Osteoporos. Int. 2014;25:2035–45.
94. Farr JN, Lee VR, Blew RM, Lohman TG, Going SB. Quantifying bone-relevant activity and its relation to bone strength in girls. Med. Sci. Sports Exerc. 2011;43:476–83.
95. Kindler JM, Ross HL, Laing EM, Modlesky CM, Pollock NK, Baile CA, et al. Load-Specific Physical Activity Scores are Related to Tibia Bone Architecture. Int J Sport Nutr Exerc Metab [Internet]. 2014;136–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25029040
96. Katzman DK, Bachrach LK, Carter DR, Marcus R. Clinical and anthropometric correlates of bone mineral acquisition in healthy adolescent girls. J. Clin. Endocrinol. Metab. 1991;73:1332–9.
97. Pearson D, Horton B, Green DJ. Cross Calibration of DXA as Part of an Equipment Replacement Program. J. Clin. Densitom. 2006;9:287–94.
98. Pocock NA, Sambrook PN, Nguyen T, Kelly P, Freund J, Eisman JA. Assessment of Spinal and Femoral Bone Density by Dual X-Ray Absorptiometry : Comparison of Lunar and Hologic Instruments. 1992;7:1081–4.
99. Article O. Discrepancies in Normative Data between Lunar and Hologic DXA Systems. Osteoporos Int. 1996;6:432–6.
100. Hagiwara S, Yang KES, Dhillon MS, Guglielmi G, Nelson DL, Genant HK. Dual X-ray Absorptiometry Forearm Software : Accuracy and Intermachine Relationship. J. Bone Miner. Res. 2000;9:1425–7.
101. Darelid A, Ohlsson C, Nilsson M, Kindblom JM, Mellstr??m D, Lorentzon M. Catch up in bone acquisition in young adult men with late normal puberty. J. Bone Miner. Res. 2012;27:2198–207.
102. Baxter-Jones ADG, Burrows M, Bachrach LK, Lloyd T, Petit M, Macdonald H, et

al. International longitudinal pediatric reference standards for bone mineral content. Bone [Internet]. Elsevier Inc.; 2010;46:208–16. Available from: http://dx.doi.org/10.1016/j.bone.2009.10.017
103. Bliuc D, Nguyen ND, Milch VE, Nguyen T V, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA [Internet]. 2009;301:513–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19190316
104. Vicente-Rodriguez G, Dorado C, Perez-Gomez J, Gonzalez-Henriquez JJ, Calbet JAL. Enhanced bone mass and physical fitness in young female handball players. Bone. 2004;35:1208–15.
105. Gómez-Bruton A, Gónzalez-Agüero A, Gómez-Cabello A, Casajús JA, Vicente-Rodríguez G. Is Bone Tissue Really Affected by Swimming? A Systematic Review. PLoS One. 2013;8.
106. Warren M, Stich A. Exercise and female adolescents: effects on the reproductive and skeletal systems. J Am Med Women's Assoc. 1998;54:115–20.
107. Torstveit MK, Sundgot-Borgen J. The female athlete triad: Are elite athletes at increased risk? Med. Sci. Sports Exerc. 2005;37:184–93.
108. Abraham S. Eating and weight controlling behaviours of young ballet dancers. Psychopathology. 1996;29:218–22.
109. Amorim T, Wyon M, Machado JC, Metsios GS, Flouris D, Koutedakis Y. Prevalence of Low Bone Mineral Density in Female Dancers. Sport. Med. 2015;45:257–68.
110. Ma NS, Gordon CM. Pediatric osteoporosis: Where are we now? J. Pediatr. [Internet]. Mosby, Inc.; 2012;161:983–90. Available from: http://dx.doi.org/10.1016/j.jpeds.2012.07.057
111. Morris FL, Naughton G a, Gibbs JL, Carlson JS, Wark JD. Prospective ten-month exercise intervention in premenarcheal girls: positive effects on bone and lean mass. J. Bone Miner. Res. 1997;12:1453–62.
112. Bradney M, Pearce G, Naughton G, Sullivan C, Bass S, Beck T, et al. Moderate exercise during growth in prepubertal boys: changes in bone mass, size, volumetric density, and bone strength: a controlled prospective study. J. Bone Miner. Res. [Internet]. 1998;13:1814–21. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9844098&retmode=ref&cmd=prlinks%5Cnpapers3://publication/doi/10.1359/jbmr.1998.13.12.1814
113. McKay HA, Petit MA, Bailey DA, Wallace WM, Schutz RW, Khan KM. Analysis of proximal femur DXA scans in growing children: comparisons of different protocols for cross-sectional 8-month and 7-year longitudinal data. J. Bone Miner. Res. 2000;15:1181–8.
114. Kröger H, Kotaniemi a, Kröger L, Alhava E. Development of bone mass and bone density of the spine and femoral neck--a prospective study of 65 children and adolescents. Bone Miner. 1993;23:171–82.
115. Richards J, Rivadeneira F, Inouye M, Pastinen T, Soranzo N, Wilson S, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008;371:1505–12.
116. Chevalley T, Bonjour JP, Ferrari S, Rizzoli R. The influence of pubertal timing on bone mass acquisition: A predetermined trajectory detectable five years before menarche. J. Clin. Endocrinol. Metab. 2009;94:3424–31.
117. Mughal MZ, Khadilkar A V. The accrual of bone mass during childhood and puberty. Curr. Opin. Endocrinol. Diabetes. Obes. [Internet]. 2011;18:28–32. Available

from: http://www.ncbi.nlm.nih.gov/pubmed/21102321
118. Harvey N, Dennison E, Cooper C. Osteoporosis: impact on health and economics. Nat. Rev. Rheumatol. [Internet]. Nature Publishing Group; 2010;6:99–105. Available from: http://dx.doi.org/10.1038/nrrheum.2009.260
119. Kanis JA, Borgström F, Compston J, Dreinhöfer K, Nolte E, Jonsson L, et al. SCOPE: A scorecard for osteoporosis in Europe. Arch. Osteoporos. 2013;8.
120. Bianchi ML, Baim S, Bishop NJ, Gordon CM, Hans DB, Langman CB, et al. Official positions of the International Society for Clinical Densitometry (ISCD) on DXA evaluation in children and adolescents. Pediatr. Nephrol. 2010;25:37–47.
121. Compston J, Cooper A, Cooper C, Francis R, Kanis JA, Marsh D, et al. Guidelines for the diagnosis and management of osteoporosis in postmenopausal women and men from the age of 50 years in the UK. Maturitas. 2009;62:105–8.
122. Scofield KL, Hecht S. Bone health in endurance athletes: runners, cyclists, and swimmers. Curr Sport. Med Rep [Internet]. 2012;11:328–34. Available from: http://graphics.tx.ovid.com/ovftpdfs/FPDDNCGCAGMFHK00/fs047/ovft/live/gv024/00149619/00149619-201211000-00014.pdf
123. Pollock N, Grogan C, Perry M, Pedlar C, Cooke K, Morrissey D, et al. Bone-mineral density and other features of the female athlete triad in elite endurance runners: A longitudinal and cross-sectional observational study. Int. J. Sport Nutr. Exerc. Metab. 2010;20:418–26.
124. Cobb KL, Bachrach LK, Greendale G, Marcus R, Neer RM, Nieves J, et al. Disordered eating, menstrual irregularity, and bone mineral density in female runners. Med. Sci. Sports Exerc. 2003;35:711–9.
125. Practice T, Medicine R. Current evaluation of amenorrhea. Fertil. Steril. [Internet]. American Society for Reproductive Medicine; 2004;82:266–72. Available from: http://dx.doi.org/10.1016/j.fertnstert.2008.08.038
126. Dimitriou L, Weiler R, Lloyd-Smith R, Turner A, Heath L, James N, et al. Bone mineral density, rib pain and other features of the female athlete triad in elite lightweight rowers. BMJ Open [Internet]. 2014;4:e004369. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3927798&tool=pmcentrez&rendertype=abstract
127. Peel N. Disorders of bone metabolism. Surg. (United Kingdom) [Internet]. Elsevier Ltd; 2015;33:15–20. Available from: http://dx.doi.org/10.1016/j.mpsur.2014.10.007
128. Shaw NJ. Management of osteoporosis in children. Eur. J. Endocrinol. 2008;159:33–9.
129. Fredericson M, Chew K, Ngo J, Cleek T, Kiratli J, Cobb K. Regional bone mineral density in male athletes: a comparison of soccer players, runners and controls. Br. J. Sports Med. [Internet]. 2007;41:664–8; discussion 668. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2465163&tool=pmcentrez&rendertype=abstract
130. Becker S, Ogon M. Epidemiology of osteoporosis. Balloon Kyphoplasty. 2008;16:1–3.
131. Vainionpää A, Korpelainen R, Leppäluoto J, Jämsä T. Effects of high-impact exercise on bone mineral density: A randomized controlled trial in premenopausal women. Osteoporos. Int. 2005;16:191–7.
132. Bonewald LF. The amazing osteocyte. J. Bone Miner. Res. 2011;26:229–38.
133. Heinonen A, Sievänen H, Kannus P, Oja P, Pasanen M, Vuori I. High-impact exercise and bones of growing girls: A 9-month controlled trial. Osteoporos. Int.

2000;11:1010–7.
134. Gennari L, Brandi ML. Genetics of male osteoporosis. Calcif. Tissue Int. 2001;69:200–4.
135. El Hage RP, Courteix D, Benhamou C-L, Jacob C, Jaffré C. Relative importance of lean and fat mass on bone mineral density in a group of adolescent girls and boys. Eur. J. Appl. Physiol. 2009;105:759–64.
136. Ilich JZ, Kerstetter JE. Nutrition in Bone Health Revisited : A Story. 2000;19:715–37.
137. Ekegrena C, Questedb R, Brodrick A. Incidence in pre-professional ballet dancers: incidence, characteristics and consequences. J. Sci. Med. Sport. 2014;17:271–5.
138. Jay FXMM. Disorders of bone remodeling. Annu Rev Pathol. 2011;6:121–45.
139. Sharan K, Yadav VK. Hypothalamic control of bone metabolism. Best Pract. Res. Clin. Endocrinol. Metab. [Internet]. Elsevier Ltd; 2014;28:713–23. Available from: http://www.sciencedirect.com/science/article/pii/S1521690X14000554
140. Locatelli V, Bianchi VE. Effect of GH/IGF-1 on Bone Metabolism and Osteoporsosis. Int. J. Endocrinol. [Internet]. 2014;2014:1–25. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4132406&tool=pmcentrez&rendertype=abstract
141. Ueland T. Bone metabolism in relation to alterations in systemic growth hormone. Growth Horm. IGF Res. 2004;14:404–17.
142. Canalis E, Agnusdei D. Insulin-like growth factors and their role in osteoporosis. Calcif. Tissue Int. [Internet]. 1996;58:133–4. Available from: http://link.springer.com/10.1007/BF02526877
143. Perrini S, Laviola L, Carreira MC, Cignarelli A, Natalicchio A, Giorgino F. The GH/IGF1 axis and signaling pathways in the muscle and bone: Mechanisms underlying age-related skeletal muscle wasting and osteoporosis. J. Endocrinol. 2010;205:201–10.
144. Murray PG, Clayton PE. Endocrine control of growth. Am. J. Med. Genet. Part C Semin. Med. Genet. 2013;163:76–85.
145. Pigeon P, Oliver I, Charlet JP, Rochiccioli P. Intensive Dance Practice Repercussions Growth and Puberty. Am. J. Sports Med. 1997;25:243–7.
146. Burckhardt P, Wynn E, Krieg M-A, Bagutti C, Faouzi M. The effects of nutrition, puberty and dancing on bone density in adolescent ballet dancers. J. Dance Med. Sci. 2011;15:51–60.
147. The Section on Endocrinology. Bone Densitometry in Children and Adolescents. Pediatrics [Internet]. 2011;127:189–94. Available from: http://pediatrics.aappublications.org/cgi/doi/10.1542/peds.2010-2961%5Cnhttp://pediatrics.aappublications.org/content/127/1/189.full.pdf
148. Nelson DA, Koo WWK. Interpretation of absorptiometric bone mass measurements in the growing skeleton: Issues and limitations. Calcif. Tissue Int. 1999;65:1–3.
149. Markou KB, Theodoropoulou A, Tsekouras A, Vagenakis AG, Georgopoulos NA. Bone acquisition during adolescence in athletes. Ann. N. Y. Acad. Sci. 2010;1205:12–6.
150. Mohan S, Richman C, Guo R, Amaar Y, Donahue LR, Wergedal J, et al. Insulin-like growth factor regulates peak bone mineral density in mice by both growth hormone-dependent and -independent mechanisms. Endocrinology. 2003;144:929–36.
151. Crockett JC, Rogers MJ, Coxon FP, Hocking LJ, Helfrich MH. Bone remodelling

at a glance. J. Cell Sci. 2011;124:991–8.
152. Delgado-Calle J. Do Epigenetic Marks Govern Bone Mass and Homeostasis? Curr. Genomics. 2012;13:252–63.
153. Jepsen KJ. Systems analysis of bone. 2009;73–88.
154. Xing W, Baylink D, Kesavan C, Hu Y, Kapoor S, Chadwick RB, et al. Global gene expression analysis in the bones reveals involvement of several novel genes and pathways in mediating an anabolic response of mechanical loading in mice. J. Cell. Biochem. 2005;96:1049–60.
155. Suuriniemi M, Mahonen A, Kovanen V, Alén M, Lyytikäinen A, Wang Q, et al. Association between exercise and pubertal BMD is modulated by estrogen receptor alpha genotype. J. Bone Miner. Res. 2004;19:1758–65.
156. Ducher G, Prouteau S, Courteix D, Benhamou C-L. Cortical and trabecular bone at the forearm show different adaptation patterns in response to tennis playing. J. Clin. Densitom. [Internet]. 2004;7:399–405. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15618600
157. Barrack MT, Van Loan MD, Rauh MJ, Nichols JF. Body mass, training, menses, and bone in adolescent runners: A 3-yr follow-up. Med. Sci. Sports Exerc. 2011;43:959–66.
158. Crabtree NJ, Arabi A, Bachrach LK, Fewtrell M, El-Hajj Fuleihan G, Kecskemethy HH, et al. Dual-energy x-ray absorptiometry interpretation and reporting in children and adolescents: The revised 2013 ISCD pediatric official positions. J. Clin. Densitom. [Internet]. Elsevier Ltd; 2014;17:225–42. Available from: http://dx.doi.org/10.1016/j.jocd.2014.01.003
159. Global Recommendations on Physical Activity for Health WHO Library Cataloguing-in-Publication Data Global recommendations on physical activity for health. [cited 2016 Oct 14]; Available from: http://apps.who.int/iris/bitstream/10665/44399/1/9789241599979_eng.pdf
160. Scofield KL, Hecht S. Bone Health in Endurance Athletes. Curr. Sports Med. Rep. [Internet]. 2012;11:328–34. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00149619-201211000-00014
161. Torstveit MK, Sundgot-Borgen J. The female athlete triad exists in both elite athletes and controls. Med. Sci. Sports Exerc. 2005;37:1449–59.
162. Warren MP, Perlroth NE. The effects of intense exercise on the female reproductive system. J. Endocrinol. 2001;170:3–11.
163. Bertelloni S, Ruggeri S, Baroncelli GI. Effects of sports training in adolescence on growth, puberty and bone health. Gynecol. Endocrinol. 2006;22:605–12.
164. Becker S, Ogon M. Epidemiology of osteoporosis. Balloon Kyphoplasty [Internet]. Elsevier Ltd; 2008;22:1–3. Available from: http://dx.doi.org/10.1016/j.beem.2008.06.001
165. Kesavan C, Mohan S, Srivastava AK, Kapoor S, Wergedal JE, Yu H, et al. Identification of genetic loci that regulate bone adaptive response to mechanical loading in C57BL/6J and C3H/HeJ mice intercross. Bone. 2006;39:634–43.
166. Tu X, Rhee Y, Condon KW, Bivi N, Allen MR, Dwyer D, et al. Sost downregulation and local Wnt signaling are required for the osteogenic response to mechanical loading. Bone [Internet]. Elsevier B.V.; 2012;50:209–17. Available from: http://dx.doi.org/10.1016/j.bone.2011.10.025
167. Tobias JH. At the crossroads of skeletal responses to estrogen and exercise. Trends Endocrinol. Metab. 2003;14:441–3.
168. Lee K, Lanyon L. Mechanical loading influences bone mass through estrogen

receptor [alpha]. 2004;64–8. Available from: http://discovery.ucl.ac.uk/183482/
169. Monroe DG, McGee-Lawrence ME, Oursler MJ, Westendorf JJ. Update on Wnt signaling in bone cell biology and bone disease. Gene [Internet]. Elsevier B.V.; 2012;492:1–18. Available from: http://dx.doi.org/10.1016/j.gene.2011.10.044
170. Liedert A, Wagner L, Seefried L, Ebert R, Jakob F, Ignatius A. Estrogen receptor and Wnt signaling interact to regulate early gene expression in response to mechanical strain in osteoblastic cells. Biochem. Biophys. Res. Commun. [Internet]. Elsevier Inc.; 2010;394:755–9. Available from: http://dx.doi.org/10.1016/j.bbrc.2010.03.065
171. Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, et al. The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. J. Biol. Chem. 2006;281:23698–711.
172. Li H, Chen D, Zhang J. Analysis of Intron Sequence Features Associated with Transcriptional Regulation in Human Genes. PLoS One. 2012;7:1–9.
173. Majewski J, Majewski J, Ott J, Ott J. Distribution and characterization of regulatory elements in the human genome. Genome Res. 2002;12:1827–36.
174. Nott A, Meislin SH, Moore MJ. A quantitative analysis of intron effects on mammalian gene expression A quantitative analysis of intron effects on mammalian gene expression. Rna. 2003;607–17.
175. Agueda L, Bustamante M, Jurado S, Garcia-Giralt N, Ciria M, Saló G, et al. A haplotype-based analysis of the LRP5 gene in relation to osteoporosis phenotypes in Spanish postmenopausal women. J. Bone Miner. Res. 2008;23:1954–63.
176. Li WF, Hou SX, Yu B, Li MM, Férec C, Chen JM. Genetics of osteoporosis: Accelerating pace in gene identification and validation. Hum. Genet. 2010;127:249–85.
177. Gennari L, Merlotti D, De Paola V, Calabrò A, Becherini L, Martini G, et al. Estrogen receptor gene polymorphisms and the genetics of osteoporosis: A HuGE review. Am. J. Epidemiol. 2005;161:307–20.
178. Xu X-H, Dong S-S, Guo Y, Yang T-L, Lei S-F, Papasian CJ, et al. Molecular genetic studies of gene identification for osteoporosis: the 2009 update. Endocr. Rev. 2010;31:447–505.
179. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, et al. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J. 2003;22:6267–76.
180. Hinton PS, Nigh P, Thyfault J. Serum sclerostin decreases following 12months of resistance- or jump-training in men with low bone mass. Bone [Internet]. Elsevier Inc.; 2016; Available from: http://linkinghub.elsevier.com/retrieve/pii/S8756328216303015
181. Ardawi M-SM, Rouzi AA, Qari MH. Physical activity in relation to serum sclerostin, insulin-like growth factor-1, and bone turnover markers in healthy premenopausal women: A cross-sectional and a longitudinal study. J. Clin. Endocrinol. Metab. [Internet]. 2012;97:3691–9. Available from: http://linksource.ebsco.com/ls.941a0f3a-fc44-412f-81a4-365a06658f2f.false/linking.aspx?&sid=OVID:embase&id=pmid:&id=doi:10.1210/jc.2011-3361&issn=0021-972X&isbn=&volume=97&issue=10&spage=3691&pages=3691-3699&date=2012&title=Journal+of+Clinical+Endocrinolo
182. Braidman IP, Hainey L, Batra G, Selby PL, Saunders PT, Hoyland J a. Localization of estrogen receptor beta protein expression in adult human bone. J. Bone Miner. Res. [Internet]. 2001;16:214–20. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/11204421
183. Vidal O, Kindblom LG, Ohlsson C. Expression and localization of estrogen receptor-beta in murine and human bone. J Bone Min. Res [Internet]. 1999;14:923–9. Available from: http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=10352100
184. Robinson TL, Snow-Harter C, Taaffe DR, Gillis D, Shaw J, Marcus R. Gymnasts exhibit higher bone mass than runners despite similar prevalence of amenorrhea and oligomenorrhea. J. Bone Miner. Res. [Internet]. 1995;10:26–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7747628
185. Maïmoun L, Coste O, Mariano-Goulart D, Galtier F, Mura T, Philibert P, et al. In peripubertal girls, artistic gymnastics improves areal bone mineral density and femoral bone geometry without affecting serum OPG/RANKL levels. Osteoporos. Int. 2011;22:3055–66.
186. Maïmoun L, Coste O, Philibert P, Briot K, Mura T, Galtier F, et al. Peripubertal female athletes in high-impact sports show improved bone mass acquisition and bone geometry. Metabolism. [Internet]. Elsevier Inc.; 2013;62:1088–98. Available from: http://dx.doi.org/10.1016/j.metabol.2012.11.010
187. Kirchner EM, Lewis RD, O´Connor PJ. Bone mineral density and dietary intake of female college gymnasts. Med. Sci. Sport. Exerc. 1994;27:543–9.
188. Di Pietro L, Stachenfeld N. The myth of the female athlete triad. Br. J. Sports Med. 2006;40:490–3.
189. Liu F, Kohlmeier S, Wang C-Y. Wnt signaling and skeletal development. Cell. Signal. 2008;20:999–1009.
190. Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, et al. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. J. Biol. Chem. 2008;283:5866–75.
191. Lin C, Jiang X, Dai Z, Guo X, Weng T, Wang J, et al. Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signaling. J. Bone Miner. Res. 2009;24:1651–61.
192. Jürimäe J, Tillmann V, Cicchella A, Stefanelli C, Võsoberg K, Tamm AL, et al. Increased sclerostin and preadipocyte factor-1 levels in prepubertal rhythmic gymnasts: associations with bone mineral density, body composition, and adipocytokine values. Osteoporos. Int. 2016;27:1239–43.
193. Waldrop J. Early identification and interventions for female athlete triad. J. Pediatr. Heal. Care. 2005;19:213–20.
194. Papanek PE. The female athlete triad: an emerging role for physical therapy. J Orthop Sport. Phys Ther [Internet]. 2003;33:594–614. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14620789
195. Pantano KJ. Strategies used by physical therapists in the U.S. for treatment and prevention of the female athlete triad. Phys. Ther. Sport [Internet]. Elsevier Ltd; 2009;10:3–11. Available from: http://dx.doi.org/10.1016/j.ptsp.2008.09.001
196. Ducher G, Turner AI, Kukuljan S, Pantano KJ, Carlson JL, Williams NI, et al. Obstacles in the optimization of bone health outcomes in the female athlete triad. Sport. Med. [Internet]. 2011;41:587–607. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-79959447480&partnerID=40&md5=f23c48fe4cf4a9d0df02920cfd236658
197. Kleposki RW. The female athlete triad: a terrible trio implications for primary care. J Am Acad Nurse Pr. 2002;14:23–6.

APPENDIX 1: Ethical approval from the NHS, UK

NHS
Health Research Authority

National Research Ethics Service

NRES Committee West Midlands - The Black Country
HRA NRES Centre Manchester
3rd Floor, Barlow House
4 Minshull Street
Manchester
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22 January 2014

Professor Yiannis Koutedakis
Professor in Exercise Physiology; Visiting Prof. in Applied Physiology, School of Sport, Performing Arts & Leisure
School of Sport & Exercise Sciences, Thessaly University, Greece; Wolverhampton University, UK
School of Sport, Performing Arts and Leisure
Walsall Campus
Gorway Road Walsall
WS13BD

Dear Professor Koutedakis

Study title:	**Prevalence of Osteoporosis and Low Bone Mineral Density in Professional Dancers**
REC reference:	**14/WM/0008**
IRAS project ID:	**146123**

Thank you for your letter of 11 January 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager, Miss Shehnaz Ishaq, nrescommittee.westmidlands-blackcountry@nhs.net

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Health Research Authority

National Research Ethics Service

NRES Committee West Midlands - The Black Country
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22 January 2014

Professor Yiannis Koutedakis
Professor in Exercise Physiology; Visiting Prof. in Applied Physiology, School of Sport, Performing Arts & Leisure
School of Sport & Exercise Sciences, Thessaly University, Greece; Wolverhampton University, UK
School of Sport, Performing Arts and Leisure
Walsall Campus
Gorway Road Walsall
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Dear Professor Koutedakis

Study title:	**Bone Health of Vocational Dance Students**
REC reference:	**14/WM/0009**
IRAS project ID:	**145975**

Thank you for your letter of 11 January 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager, Miss Shehnaz Ishaq, nrescommittee.london-fulham@nhs.net

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, **subject to the conditions specified below.**

APPENDIX 2: Ethical approval from the IRS, Portugal

Exma. Sr.ª
Dr.ª Tânia Patricia Amorim
tania_amorim@hotmail.com

C/C:

Sua Referência	Sua Comunicação de	Nossa Referência	Data
		4105 /CES	09-04-2013

Assunto: "Bone Health of Ballet Dancers: a Multifaceted Approach"

Proc.063/CES/INV/2012: Parecer da Comissão de Ética – Secção de Investigação

O Projecto com a designação **"Bone Health of Ballet Dancers: a Multifaceted Approach",** foi sujeito à apreciação da Comissão de Ética para a Saúde da ARSLVT (Secção de Investigação) na sua reunião de 05-04-2013, tendo merecido Parecer favorável.

Conflito de interesses: não identificados.

O Conselho Directivo, atento ao teor do Parecer emitido por aquela Comissão, entende estarem reunidas as condições para a sua concretização.

Com os melhores cumprimentos,

O Vice - Presidente do Conselho Directivo

Luis Pisco

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APPENDIX 3: Written consent form UK

Participant Number:

CONSENT FORM – DANCE ADULTS

Title of Project: **Osteoporosis and Low Bone Mineral Density in Professional Dancers**

Name of Researcher: **Prof. Yiannis Koutedakis**

Please initial all boxes

1. I confirm that I have read and understand the information sheet dated **[05/12/2013]** (version **[5.0]**) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to take part in the above study. ☐

4. "I understand that relevant data collected during the study may be looked at by individuals from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to this data" ☐

Name of Participant	Date	Signature
Name of Person taking consent.	Date	Signature

APPENDIX 4: Written consent form Portugal

U.PORTO

DECLARAÇÃO DE CONSENTIMENTO

Estudo: Saúde Óssea da Bailarina e Bailarino

Considerando a "Declaração de Helsínquia" da Associação Médica Mundial (Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)

Eu, abaixo-assinado, (nome completo) _____ _____, compreendi a explicação que me foi fornecida acerca da investigação que se tenciona realizar. Foi-me dada a oportunidade de fazer as perguntas que julguei necessárias e, no caso de as ter feito, de todas obtive resposta satisfatória.

Tomei conhecimento de que, de acordo com as recomendações da declaração de Helsínquia, a informação ou explicação que me foi prestada versou os objectivos, os métodos, os benefícios previstos, os riscos potenciais e o eventual desconforto. Além disso, foi-me afirmado que tenho o direito de recusar a todo o tempo a sua participação no estudo.

Foi-me igualmente explicado que todos os dados obtidos serão confidenciais. Apenas eu e o investigador responsável teremos acesso a todos os meus dados.

Por isso, consinto que lhe seja aplicado o método ou o inquérito proposto pelo investigador.

Data: ___ / _____ / 20__

Assinatura: _____

O Investigador responsável

Nome:_____

Assinatura:_____

APPENDIX 5: BPAQ

Bone-Specific Physical Activity Questionnaire (BPAQ)

SUBJECT ID:	DATE:

1. **Please list <u>any</u> sports or other physical activities you have participated in regularly. Please <u>tick</u> the boxes to indicate how old you were for each sport/activity and how many years you participated for.**

Activities \ Age:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Activities \ Age:	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60

Activities \ Age:	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90

2. **Please list the sports or other physical activities (be as specific as possible) you participated in regularly during the <u>last 12 months</u> and indicate the average frequency (sessions per week)?**

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

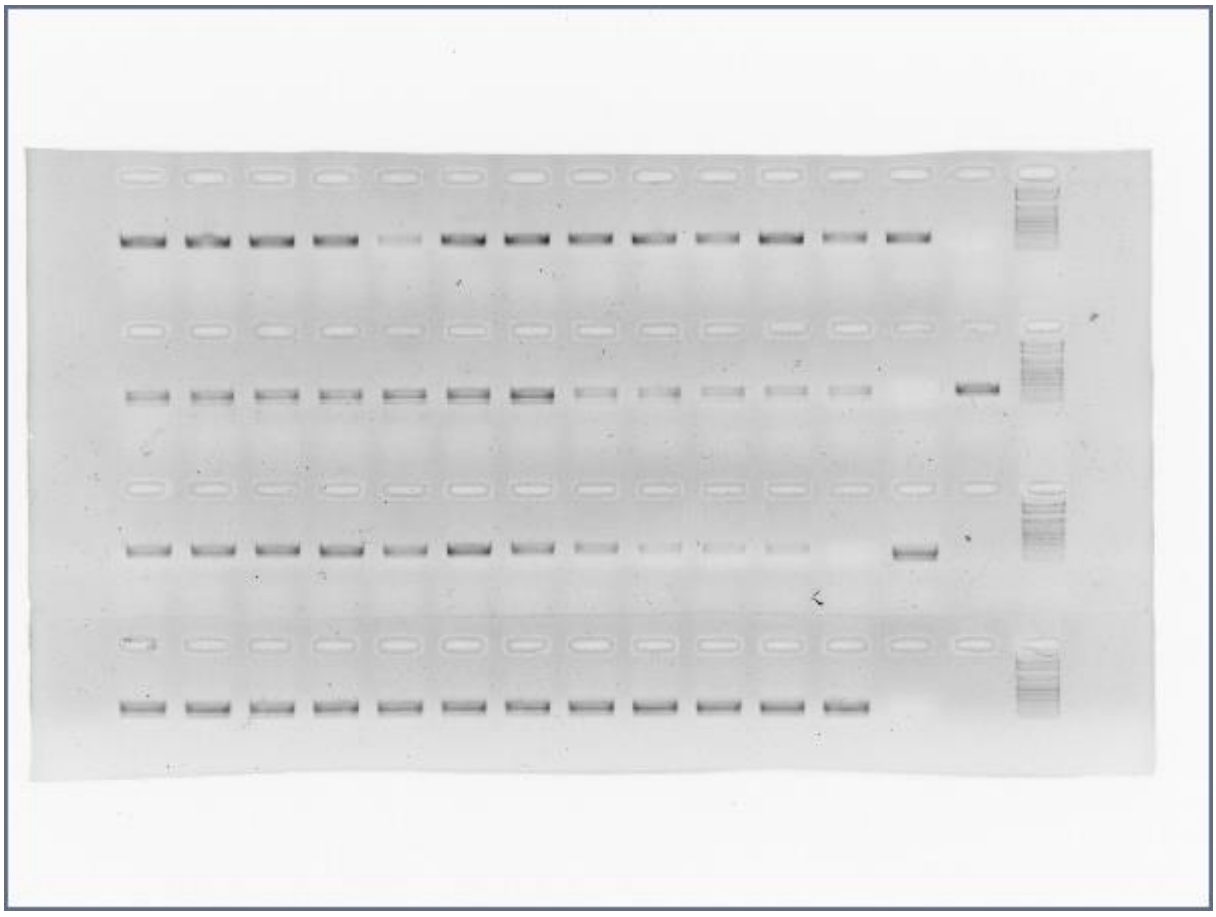
Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

APPENDIX 6: Representative image of the genotyped genes



APPENDIX 7: Supplemental tables

Table 14. General characteristics of professional ballet dancers and vocational dance students included in the study

	Professional ballet dancers		Vocational dance students	
	Low BMD	Normal BMD	Low BMD	Normal BMD
Age (yrs.)	37.5 ± 9.3*	30.5 ± 8.9	13.0 ± 1.7	12.5 ± 1.9
Weight (kg)	51.9 ± 9.4	57.1 ± 9.8	41.0 ± 8.2	41.1 ± 9.5
Height (cm)	164.3 ± 6.5	168.8 ± 7.4	155.3 ± 10.3	152.7 ± 11.1
Age menarche (yrs.)(1)	13.3 ± 1.9	13.7 ± 1.8	12.7 ± 0.8	12.4 ± 1.2
Primary amenorrhea(2)	5*	42.9	0	0
Secondary amenorrhea(2)	5.3	14.3	29.4	16.7
Oligomenorrhea(2)	15.8	0	35.3	33.3
Energy intake (Kcal/day)	1509.0 ± 403.0	1806.6 ± 597.6	1458.8 ± 426.3**	1734.6 ± 443.3
Energy availability (kcal/kgFFM/day)	26.4 ± 16.0	28.4 ± 11.9	31.6 ± 19.4*	43.6 ± 23.5
BMD FN (g/cm^2)	1.04 ± 0.16**	1.20 ± 0.15	0.78 ± 0.13**	0.96 ± 0.17
BMD LS (g/cm^2)	1.16 ± 0.16	1.25 ± 0.10	0.74 ± 0.12**	0.91 ± 0.17
BMD FA (g/cm^2)	0.76 ± 0.07**	0.85 ± 0.09	0.55 ± 0.06**	0.62 ± 0.09

Values are means ± SD

* p<0.05; ** p<0.01; *** p<0.001; statistical significant differences between groups

BMD: bone mineral density; FN: femoral neck; LS: lumbar spine; FA: forearm

(1) Means were calculated considering all females that have reached the menarche by the time of the assessment

(2) Values are percentages.

Table 15. Adjusted BMC and BMD values of the professional ballet dancers and vocational dance students included in the study

	Professional ballet dancers				Vocational dance students			
	Low BMD	IC 95%	Normal BMD	IC 95%	Low BMD	IC 95%	Normal BMD	IC 95%
BMD FN (g/cm^2)	1.08 ± 0.03	1.03 – 1.13	1.13 ± 0.04	1.05 – 1.21	0.77 ± 0.02***	0.73 – 0.81	0.98 ± 0.02	0.94 – 1.02
BMD LS (g/cm^2)	1.17 ± 0.03	1.11 – 1.23	1.22 ± 0.04	1.13 – 1.31	1.15 ± 0.03	1.08 – 1.22	1.25 ± 0.06	1.13 – 1.36
BMD FA (g/cm^2)	0.77 ± 0.01**	0.75 – 0.79	0.83 ± 0.02	0.79 – 0.86	0.54 ± 0.01***	0.52 – 0.56	0.63 ± 0.01	0.61 – 0.65

Values are means + SD
* p<0.05; ** p<0.01; *** p<0.001
BMD: bone mineral density; FN: femoral neck; LS: lumbar spine; FA: forearm
Bone parameters in professional ballet dancers were adjusted for age, sex and primary amenorrhea
Bone parameters in vocational dance students were adjusted for energy availability, fat intake, calcium intake, carbohydrate intake and sex

Table 16. General characteristics of the studied population included in the genetic analysis

	Controls (n=151)		Dancers (n=151)	
	Normal BMD Forearm	Low BMD Forearm	Normal BMD Forearm	Low BMD Forearm
N	122	29	98	50
Age (yrs.)	17.7±10.8	20.6±10.3	14.8±6.6	24.8±13.9***
Weight (kg)	56.1±13.1	51.3±11.9	43.2±10.4	46.0±10.8+++
Height (cm)	156.9± 21.2	156.9±8.9	150.7±29.0	153.3±32.9
FA BMD (g/cm^2)	0.678±0.124	0.659±0.141	0.641±0.120	0.629±0.133
LS BMD (g/cm^2)	0.931±0.180	0.904±0.204	0.904±0.209	0.928±0.257
FN BMD (g/ cm^2)	0.923±0.133	0.866±0.158	0.950±0.195	0.881±0.208
	Normal BMD LS	Low BMD LS	Normal BMD LS	Low BMD LS
N	110	40	96	53
Age (yrs.)	18.3±11.1	17.9±10.0	19.6±12.0	15.5±7.4*
Weight (kg)	55.8±14.9	54.8±14.9	44.9±10.8	42.7±9.9+++
Height (cm)	156.2±21.7	158.9±11.3	148.8±36.6	156.6±10.3
FA BMD (g/cm^2)	0.681±0.120	0.835±0.135	0.663±0.129	0.573±0.09
LS BMD (g/cm^2)	0.966±0.174	0.835±0.176	0.996±0.222	0.761±0.132
FN BMD (g/ cm^2)	0.932±0.134	0.859±0.135	0.996±0.196	0.780±0.137
	Normal BMD FN	Low BMD FN	Normal BMD FN	Low BMD FN
N	99	51	99	53
Age (yrs.)	19.9±11.9	14.9±7.1	19.0±11.4	16.6±9.3
Weight (kg)	57.0±12.7	51.6±13.1	44.4±11.1	43.2±9.4+++
Height (cm)	157.6±22.7	155.5±10.7	148.2±35.9	157.9±9.7
FA BMD (g/cm^2)	0.700±0.128	0.622±0.113	0.663±0.129	0.569±0.08
LS BMD (g/cm^2)	0.984±0.173	0.818±0.154	0.984±0.226	0.770±0.139
FN BMD (g/ cm^2)	0.960±0.125	0.819±0.114	1.001±0.187	0.763±0.108

Values are mean±SD
* p<0.05; ** p<0.01; *** p<0.001; statistically significant when comparing with dancers with normal BMD
+ p<0.05; ++ p<0.01; +++ p<0.001; statistically significant when comparing with controls with normal BMD
BMD: bone mineral density; FN: femoral neck; LS: lumbar spine; FA: forearm

Table 17. Characteristics of the genotyped SNPs.

Signalling pathway	Gene	SNP	Genomic position	Genic position	Alleles (major/minor)	MAF[a] controls	MAF dancers	HWE[b] controls
Wnt/β-catenin	LRP5	rs491347	11:68402220	Intron	A/G	26.3	30.0	0.1460
Wnt/β-catenin	LRP5	rs682429	11:68311851	Intron	A/G	34.9	32.3	0.0003
Wnt/β-catenin	LRP5	rs2508836	11:68323797	Intron	C/T	36.2	29.0	0.7260
Wnt/β-catenin	LRP5	rs587808	11:68384245	Intron	A/G	38.2	39.0	0.2278
Wnt/β-catenin	LRP5	rs312786	11:68352509	Intron	G/T	30.6	28.3	0.7061
Wnt/β-catenin	SOST	rs851054	17:43759255	Promoter	T/C	31.3	41.7	0.0080
Wnt/β-catenin	SOST	rs10534024	17:43760138	Promoter	-/TCC	30.3	37.7	0.0072
ER	ESR1	rs2234693	6:151842200	Intron	T/C	48.7	44.6	0.0237
ER	ESR1	rs9340799	6:151842246	Intron	A/G	42.1	33.7	0.3200
ER	ESR2	rs1256030	14:64280452	Intron	G/A	36.5	43.3	0.0813
ER	ESR2	rs960070	14:64278461	Intron	C/G	42.8	49.0	0.0691

[a]MAF: minor allele frequency
[b]HWE: Hardy-Weinberg Equilibrium (significance cut off $p<0.01$)

Table 20. Haplotype association test

Gene	Haplotype	Dancers normal BMD versus dancers low BMD								
		Forearm	OR (CI)	*p*	LS	OR (CI)	*p*	FN	OR (CI)	*p*
LRP5	ACAG*	0.432	1.00		0.429	1.00			1.00	
	ACAT	NA	NA	NA	0.012	4.28(0.29-64.06)	0.293	0.424	3.69(0.28-48.29)	0.319
	ACGG	0.075	3.16(1.03-9.70)	0.044	0.079	1.31(0.42-4.07)	0.639	0.013	0.96(0.29-3.23)	0.949
	ACGT	0.012	3.33(0.19-58.42)	0.411	NA	NA	NA	0.079	NA	NA
	ATAG	0.031	0.99(0.15-6.58)	0.994	0.034	1.61(0.35-7.53)	0.542	NA	1.37(0.23-8.12)	0.726
	ATAT	0.073	1.09(0.28-4.18)	0.900	0.072	0.47(0.11-2.01)	0.308	0.029	0.70(0.18-2.75)	0.615
	ATGG	NA	NA	NA	0.019	0.10(0.00-311-48)	0.575	0.076	0.18(0.00-17.23)	0.461
	ATGT	0.041	5.20(0.96-28.26)	0.056	0.039	0.79(0.13-4.57)	0.788	0.020	2.35(0.48-11.63)	0.294
	GCAG	0.044	2.63(0.74-9.37)	0.137	0.047	1.33(0.35-5.04)	0.676	0.039	0.73(0.17-3.17)	0.674
	GCGG	0.097	1.33(0.47-3.70)	0.590	0.095	0.61(0.20-1.86)	0.384	0.051	0.58(0.18-1.87)	0.361
	GCGT	0.029	2.53(0.49-13.06)	0.268	0.028	6.25(0.86-45.44)	0.070	0.027	**8.97(1.14-70.31)**	**0.037**
	GTGT	0.110	1.13(0.42-3.03)	0.807	0.113	0.94(0.38-2.30)	0.891	0.115	0.96(0.38-2.42)	0.932
ESR1	TA*	0.536	1.00		0.539	1.00		0.543	1.00	
	CA	0.123	1.62(0.80-3.31)	0.182	0.122	0.55(0.25-1.18)	0.126	0.117	1.09(0.53-2.23)	0.814
	CG	0.330	0.69(0.39-1.24)	0.216	0.327	0.57(0.32-1.00)	0.050	**0.329**	**0.53(0.29-0.96)**	**0.037**
	TG	NA	NA	NA	0.012	0.67(0.05-8.22)	0.756	0.001	0.95(0.08-11.65)	0.970
Gene	Haplotype	Controls normal BMD versus Dancers low BMD								
		Forearm	OR (CI)	*p*	LS	OR (CI)	*p*	FN	OR (CI)	*p*
LRP5	ACAG*	0.403	1.00		0.420	1.00		0.414	1.00	
	ACAT	NA	NA	NA	0.014	8.13(0.44-149.13)	0.158	0.012	5.66(0.30-106.68)	0.247
	ACGG	0.084	3.70(0.93-14.70)	0.063	0.090	1.29(0.35-4.72)	0.699	0.095	1.09(0.29-4.11)	0.903
	ACGT	0.017	1.18(0.06-24.99)	0.915	NA	NA	NA	NA	NA	NA
	ATAG	0.041	0.99(0.16-6.13)	0.995	0.057	0.83(0.18-3.85)	0.809	0.067	0.51(0.10-2.52)	0.408
	ATAT	0.061	1.46(0.35-6.20)	0.604	0.051	0.53(0.06-4.50)	0.564	0.053	0.48(0.08-2.93)	0.426
	ATGT	0.091	1.24(0.40-3.90)	0.710	0.067	0.75(0.17-3.24)	0.702	0.076	1.19(0.32-4.38)	0.798
	GCAG	**0.047**	**6.43(1.33-31.14)**	**0.021**	0.036	3.72(0.41-33.50)	0.241	0.031	11.75(1.11-124.17)	0.041
	GCGG	0.085	1.20(0.37-3.85)	0.764	0.070	1.29(0.34-4.93)	0.708	0.065	0.61(0.13-2.96)	0.539
	GCGT	0.023	4.51(0.52-38.83)	0.170	**0.026**	**12.70(1.22-132.18)**	**0.034**	0.033	4.97(0.55-44.97)	0.154
	GTGT	0.103	1.16(0.39-3.45)	0.787	0.120	0.75(0.27-2.06)	0.570	0.129	1.08(0.33-3.55)	0.899
ESR1	TA*	0.507	1.00		0.511	1.00		0.509	1.00	
	CA	0.115	1.78(0.82-3.86)	0.143	0.078	1.06(0.39-2.92)	0.907	0.113	1.10(0.44-2.74)	0.841
	CG	0.365	0.58(0.32-1.06)	0.079	**0.395**	**0.43(0.22-0.82)**	**0.011**	**0.366**	**0.39(0.19-0.80)**	**0.001**
	TG	NA	NA	NA	0.017	0.52(0.06-4.42)	0.549	0.015	0.78(0.10-5.99)	0.808

*reference haplotype
NA: not applicable due to haplotype frequency of zero in one of the groups